[illegible]HOLOGY AND PHYSIOLOGY OF AZOSPIRIL[illegible]
GROWN ON BETA-HYDROXYBUTYRATE

By

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This dissertation is dedicated to Isabel Stewart [illegible], Robert Bleakley, the only three people who can say "Of course" without my doubting them.

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TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MORPHOLOGY AND PHYSIOLOGY OF AZOSPIRILLA GROWN ON BETA-HYDROXYBUTYRATE

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Strains of Azospirillum brasilense and Azospirillum lipoferum were cultured with beta-hydroxybutyrate (BHB) to determine if they could be converted in high numbers to cyst-like forms, as can some strains of Azotobacter spp Azospirillum brasilense strain JM 125A2 grew poorly on BHB but produced some nonmotile cells of cyst-like morphology Azospirillum brasilense strain Cd grew better on BHB and often produced elongated cells as well as some nonmotile, cyst-like cells. Capsules and accumulation of poly-beta-hydroxybutyrate (PHB) were common features of all putative cysts. Encapsulation occurred with all A. lipoferum strains tested. Cells accumulated PHB and assumed elongated, filamentous shapes as they lost motility. Later, capsules were produced and microfloc formed. The filamentous cells eventually formed septa. Several cell shapes were present

[illegible] flocs, but all cells possessed intracellular [illegible] capsules. Some cells within flocs appeared cyst like. Broth studies indicated that alkaline pH does not cause these morphological changes.

Cells of Azospirillum lipoferum Sp RG6xx grown on nitrogen-free BHB agar accumulated up to 57% of their dry weight as PHB, compared to 3-6% when grown with combined nitrogen. Neither vegetative nor encapsulated cells of this strain survived in significant numbers after 8 days of desiccation. Vegetative cells of this strain multiplied several fold and retained viability during 9 days of starvation for carbon and nitrogen, whereas encapsulated cells were reduced to 25% of their original numbers. Nonmotile, encapsulated cells produced motile vegetative cells when incubated with nitrate, ammonium, or soil extract but did not do so appreciably in nitrogen free, buffered-salts solution with or without carbon sources. Treatment with Tris-EDTA did not result in expulsion of cells from their capsular coats, as it does for mature Azotobacter spp cysts. Studies with chloramphenicol indicated that encapsulated cells do not possess the enzymes needed for growth and emergence from their capsules.

The studies suggested that PHB accumulation and capsule formation during unbalanced growth precede the formation of dormant cyst-like cells.

CHAPTER I
INTRODUCTION AND EXPERIMENTAL APPROACH

Ecology of Azospirilla

Bacteria of the genus Azospirillum have been isolated from tropical soils and from the roots of cereal crops and forage grasses in several areas of the world (Dobereiner et al., 1976; Tyler et al., 1979; Lamm and Neyra, 1981). Their numbers in nonrhizosphere soil can be as high as 10^4 cells/g soil (Dobereiner, 1978), while their numbers in rhizosphere soil can be as high as 10^7 cells/g soil (Krieg and Dobereiner, 1984).

Agricultural interest in Azospirillum spp. resulted from recognition of their ability to reduce atmospheric dinitrogen. The enzyme catalyzing this reaction, nitrogenase, is inactivated in the presence of combined nitrogen or oxygen (Okon et al., 1976a). Azospirilla fix dinitrogen under microaerophilic conditions in nitrogen-free media in the laboratory (Day and Dobereiner, 1976; Okon et al., 1976a). Nonrhizosphere soil is usually too poor in available, utilizable carbon sources to enable Azospirillum spp. to fix dinitrogen, but they can do so in the more carbon-rich rhizosphere environment (Dobereiner et al., 1976). Maximum nitrogenase activity with inoculated plants

[illegible] reproduction [illegible]
the plant reviewed by Patriquin et al (1983), after plant uptake and other processes have reduced the amount of combined nitrogen in the root zone (Okon and Hardy, 1983). Sometimes low amounts of fixed nitrogen have been incorporated into plant tissue. The transfer of fixed nitrogen from bacterium to plant seems slow, probably because bacterial nitrogen is made available for plant uptake only after the mineralization of the organic nitrogen of dead bacteria (Okon et al., 1983)

Although the nitrogenase activity of Azospirillum spp may not directly provide quick or agriculturally significant benefits to inoculated plants, the bacteria have been found to possess other characteristics that may benefit plants Axenic associations of grass seedlings and Azospirillum spp have resulted in rapid proliferation of lateral roots and root hairs, probably due to bacterial production of indole-3-acetic acid and other plant growth substances (Tien et al., 1979; Umali-Garcia et al., 1980; Jain and Patriquin, 1985) It is also possible that Azospirillum spp. can enhance production of plant growth substances by the plants themselves (Kapulnik et al., 1985) In any case, associations of Azospirillum spp with plant roots have led to significant increases of commercially valuable plant components in both axenic laboratory experiments (Kapulnik et al., 1985) and field inoculations

(Okon and Hardy, 1983). Short-term axenic associations have also resulted in enhanced uptake of mineral ions by grass roots (Lin et al., 1983). This effect may be due to the influence of plant growth substances, or to softening of the middle lamellae of root cells by pectolytic bacterial enzymes, which some azospirilla are known to produce (Umali-Garcia et al., 1980; Tien et al., 1981). Such effects on root morphology and activity may make inoculation with azospirilla beneficial in some agricultural situations.

The rhizosphere environment is prone to extreme chemical and physical fluctuations (Foster and Bowen, 1982). This may lead to periods when azospirilla are inactive due to environmental limitations. Cells of azospirilla can vary morphologically (Krieg and Dobereiner, 1984). Some of these cell forms may be dormant or resting stages, in which activities of possible benefit to plants are not expressed. Pleomorphic forms of azospirilla usually possess capsules, and contain large amounts of the reserve polymer poly-β-hydroxybutyrate (PHB). This study describes attempts to obtain such forms in high numbers by laboratory culture. The general topics of capsules, PHB, physiological dormancy, and desiccation resistance are directly related to this study, and will be briefly reviewed in this introduction after discussion of some key aspects of *Azospirillum* spp. physiology.

Physiology of Azospirilla

The three species in the genus Azospirillum all [illegible] mainly respiratory type of metabolism. They fix dinitrogen in microaerophilic environments where combined nitrogen concentration is low, and utilizable carbon-and-energy sources are available. When provided with metabolizable carbon, along with ammonium, nitrate, or other combined nitrogen sources, they can grow under aerobic conditions. In either situation, they grow well on the salts of organic acids such as malate, succinate, lactate, or pyruvate (Krieg and Dobereiner, 1984). Azospirillum brasilense can use some carbohydrates, including fructose, galactose, and arabinose. Azospirillum lipoferum is also able to use these sugars, as well as glucose, mannose, and sorbose (Martinez-Drets et al., 1984). The most recently recognized species, Azospirillum amazonense, differs from the other two species in that it can grow on sucrose and other disaccharides (Martinez-Drets et al., 1985). Both A. brasilense and A. amazonense can synthesize their own biotin, whereas A. lipoferum can only grow if exogenous biotin is available (Falk et al., 1985).

Microaerophilic culture conditions for dinitrogen fixation can be established by culturing azospirilla in media containing 0.05% (wt/vol) agar. The bacteria grow and form a pellicle slightly below the agar surface, where diffusion of O_2 from the culture-vessel headspace balances the

uptake of O_2 by the bacteria, allowing both cell respiration and protection of the oxygen-sensitive nitrogenase (Okon et al , 1976a) Broth cultures can fix dinitrogen if the dissolved oxygen level is well controlled (Okon et al , 1976a) The bacteria are also able to grow on the surface of nitrogen-free, aerobically incubated agar plates (Day and Dobereiner, 1976).

The flagella of azospirilla enable them to move to whatever sites their physiological state demands They have been shown to exhibit aerotaxis to microaerophilic sites (Barak et al , 1982) Alternatively, cells may aggregate, thereby creating a microaerophilic environment by the respiration of many cells in a small space (Barak et al., 1982). The grass rhizosphere may contain microaerophilic sites (reviewed by Patriquin et al , 1983) Azospirilla could migrate from soil toward such sites, where nitrogenase activity could subsequently be expressed

The respiratory metabolism of Azospirillum spp- includes the ability of many strains to denitrify, reducing nitrate or nitrite to more reduced nitrogenous compounds under anaerobic conditions if enough metabolizable carbon source is available (Neyra and Dobereiner, 1977 Neyra and van Berkum, 1977 Nelson and Knowles, 1978). Under certain laboratory conditions, denitrification has been shown to provide enough ATP to support anaerobic growth of azospirilla (Rothe et al , 1981; Zimmer et al , 1984). The

ATP derived from [illegible] dehydrogenase activity (Scott et al., 1979). But it seems probable that dinitrogen fixation under these conditions can support growth of the bacteria (Bothe et al., 1981). Recent work by Neuer et al. (1985) has shown that, in axenic wheat-Azospirillum spp. associations, both dinitrogen fixation and denitrification can occur.

Morphology of Azospirilla

Azospirilla are Gram-negative bacteria (Tarrand et al., 1978). The structural layers external to the cytoplasmic membrane of Gram-negative prokaryotes have been reviewed (Costerton et al., 1974). Depending on cultural conditions and the bacterial strain, polysaccharide or capsular layers may be present as the outermost layers of the cell.

A growing Gram-negative cell divides by binary fission to produce two daughter cells of approximately equal size. Division begins with invagination of the cytoplasmic membrane and peptidoglycan, until a complete transverse septum or cross wall is formed. When the septum is completely formed and cleaved, the two daughter cells separate (Leive and Davis, 1980). As will be discussed later, this cell division process can be disrupted, resulting in formation of filaments or chains, which accounts partially for pleomorphism of azospirilla.

Cells of Azospirillum brasilense and Azospirillum lipoferum have a similar appearance when cultured in broth containing combined nitrogen. They are short, plump, slightly curved rods averaging 1.0 µm in diameter and 2.1 to 3.8 µm in length. They are motile in broth by means of a single, polar flagellum (Tarrand et al., 1978).

Cells of azospirilla often contain granules of the polymer PHB (Krieg and Dobereiner, 1984). Grown with combined nitrogen, Azospirillum brasilense Sp 7 (ATCC 29145) has 0.5% to 1.0% of its dry weight as PHB. When grown in dinitrogen-fixing conditions, the PHB content rises until as much as 25.0% of its dry weight is PHB (Okon et al., 1976b). Granules of PHB are present in cells grown on combined nitrogen, but granule size and number are reduced compared to that found in dinitrogen-fixing cells (Albrecht and Okon, 1980).

Under certain cultural conditions, cells of azospirilla produce an outermost layer of capsular polysaccharide. When grown on an agar medium containing peptone, succinic acid and ammonium sulfate at 37°C for 48 to 72 hours, a small proportion of cells are Gram-variable, possibly because they possess capsules. On this medium, A. brasilense exhibits more Gram-variability than does A. lipoferum. When cells of either species are cultured in the broth form of this medium, they stain uniformly Gram-negative, at least in young cultures (Krieg and Dobereiner, 1984).

Prokaryotic Exopolysaccharides

Many genera of both Gram-positive and Gram-negative bacteria include species that can produce polysaccharide layers outside their cell wall. Such layers have been referred to as capsules, exopolysaccharides (Sutherland, 1977) or glycocalyces (Costerton et al., 1981). Depending on laboratory cultural conditions, these polymers can assume different forms. Slime layers adhere loosely, if at all, to the cell and can often be separated from cells by centrifugation. Capsular layers appear to be tightly bound to the cell itself, and cannot be easily separated from cells. Microcapsules are so thin that their presence outside the cell wall cannot be observed using staining and light microscopy, while macrocapsules are of sufficient width to be so resolved (Ward and Berkeley, 1980).

Although proteins are sometimes present in bacterial capsules, most capsules are mainly polysaccharide in composition. The polysaccharides are extensively hydrated, and up to 99% by weight of the capsule is accounted for as water (Costerton et al., 1981).

The ATP needed to activate sugar residues for exopolysaccharide synthesis has been shown to comprise a significant proportion of total-cellular-ATP demand for some bacteria. Even when the carbon supply is growth limiting, some strains of bacteria produce extracellular polysaccharides (Jarman and Pace, 1984).

For many bacteria in culture [illegible] carbon to nitrogen (C/N) ratio promotes capsule formation (Sutherland, 1977; Costerton et al., 1981). Some species manufacture exopolysaccharide throughout all phases of growth, while others produce it only at certain stages of growth (Sutherland, 1977). Exopolysaccharides of more than one composition can be formed by the same bacterium under different environmental conditions (Geesey, 1982).

In laboratory culture, exopolysaccharides may be nonessential for bacterial growth. Enzymatic removal of capsules often causes no reduction in viability of the decapsulated cells (Dudman, 1977). Nonencapsulated mutants may grow better in laboratory culture than do encapsulated cells, since they expend no energy for capsular synthesis (Costerton et al., 1981). Many nonencapsulated laboratory strains are mutants that have lost the ability of the wild type to produce exopolysaccharide. In other instances, common laboratory media have too low a C/N ratio to promote exopolysaccharide synthesis.

Attachment of bacteria to surfaces by their exopolysaccharides is the rule in nature, whether the surface is an inert mineral particle or a biological surface such as a plant root (Costerton et al., 1981). Natural environments are far different from laboratory cultural conditions, containing many more potential hazards to bacterial survival. In natural environments, the presence of

exopolysaccharides may aid the survival of bacteria (Dudman, 1977). Exopolysaccharides can concentrate nutrients from the surrounding solution phase. They give some bacteria increased resistance to antibiotics, surfactants, and other chemicals, as well as deterring their engulfment by phagocytic cells (Costerton et al., 1981). Other advantages of exopolysaccharides have been suggested, such as mediation of gas exchange between bacteria and their surroundings, but they have proven difficult to prove experimentally. Extracellular enzymes might also be located within or at the surface of capsules (Geesey, 1982).

Nur et al. (1980) found that A. brasilense Sp 7 and an Israeli isolate of A. brasilense both possessed small capsules discernible by electron microscopy when grown on nutrient agar. Umali-Garcia et al. (1980) found that when certain A. brasilense strains and grass seedlings were incubated together for 10 to 30 min at 30°C, many bacteria adhered to the grass roots, with granular material accumulating on the surfaces of root hairs, and fibrillar material accumulating on the surfaces of older, epidermal root cells. It is known that bacterial exopolysaccharides may appear either granular or fibrous (Foster and Bowen, 1982). The A. brasilense strains seemed to rapidly produce both types of exopolysaccharides in axenic association with grass roots. After 2 to 4 days of axenic association with grass roots, from two to four cells of A. brasilense Sp 7

were sometimes seen to be enclosed within a common envelope or capsule. Such structures were not observed when the bacteria were grown in trypticase soy broth (Umali-Garcia et al., 1980). This is another indication that the low C/N ratio of complex broth media can repress extensive capsule formation by azospirilla, while the high C/N ratio near plant roots can promote capsule formation.

Recent work by Sadasivan and Neyra (1985) verified that the forms of carbon and nitrogen made available to azospirilla can have a profound effect on exopolysaccharide synthesis. When A. brasilense Sp 7 and A. lipoferum Sp. 59b (ATCC 29707) were cultured in broth containing 8.0 mM fructose and 0.5 mM KNO_3, they grew as individual motile cells for only 6 hours and then started to clump, as exopolysaccharide production led to floc formation. Organic acids yielded fewer flocs than did sugars, and other nitrogen sources, such as ammonium, yielded fewer flocs than did nitrate. The cells in flocs appeared initially to be enmeshed in a loose, fibrillar matrix that condensed progressively over a week's time. When cells were grown, harvested by centrifugation, and resuspended in broth lacking carbon, the cells remained freely suspended. This suggests that azospirilla have a high ATP demand for exopolysaccharide synthesis. Chemical analysis showed that cellulose was a major component of the exopolysaccharide.

[illegible] are not degraded by cellulase, [illegible] more than one type of exopolysaccharide was present.

Poly-β-Hydroxybutyrate (PHB)

In a constant and favorable environment where all nutrients are present in sufficient amounts, bacteria grow for a time in a steady state, where every component of the cell culture increases by the same constant factor per unit time. This is balanced growth, and occurs during the logarithmic phase of the growth curve (Ingraham et al., 1983). If one or more nutrients become limiting, balanced growth is not maintained. When the carbon or energy supply is in excess, so that one or more other nutrients limit growth, some microorganisms respond by synthesizing and accumulating intracellular polymers having an energy-storage function (Dawes and Senior, 1973).

The cell catabolizes these polymers when the energy supply from exogenous sources is no longer sufficient to maintain processes needed for maintenance of cell viability. These processes may include osmotic regulation, maintenance of intracellular pH and transmembrane potentials, and turnover of cellular constituents such as proteins and nucleic acids. The energy required for these processes is called the energy of maintenance. Some microorganisms do not produce special energy-storage polymers. Faced with a starvation environment, they are forced to utilize their own

basal components, such as proteins and RNA, for energy sources. Possession of energy-storage polymers can benefit some species facing starvation, in that they degrade these polymers instead of or before they are forced to degrade such essential components as proteins (Dawes and Senior, 1973). However, different microorganisms utilize common constituents at different rates and in different sequences when starved. The possession of energy-reserve polymers does not spare degradation of protein and other basal components in some species during starvation. Most microorganisms that remain viable after prolonged starvation have a low endogenous metabolism, matched closely to their maintenance energy requirements. Starved microorganisms that rapidly metabolize polymers generally lose viability quickly (Dawes, 1976).

Three main types of microbial energy-storage compounds are known. Some species can accumulate more than one. All of these compounds have high molecular weights, and only a slight effect on the internal osmotic pressure of the cell. The amount of each compound a cell accumulates can vary widely, depending on environmental conditions.

Intracellular polyphosphates and glycogen-like polysaccharides are two types of energy-storage compounds formed by some eukaryotic and prokaryotic microorganisms. The synthesis of both types requires ATP.

The th[illegible] microbial energy-reserve polymer is [illegible]
ydroxybutyrate, a straight chain homopolymer of D[illegible]
ydroxybutyrate. It is found only in prokaryotic [illegible],
including both Gram-positive and Gram-negative spec[illegible]. Its
synthesis requires reducing power in the forms of NADH or
NADPH, but does not require the direct expenditure of ATP
(Dawes and Senior, 1973).

With phase contrast microscopy, large accumulations of
PHB within bacterial cells appear as light-refractile gran-
ules. A single granule may contain several thousand PHB
molecules (Dawes and Senior, 1973). Each granule is bounded
by a nonunit-membrane layer, which is probably formed from
the cytoplasmic membrane. Presumably the enzymes for
polymerization and depolymerization of PHB are present in
this membrane layer (Shively, 1974).

Many of the Azotobacteraceae accumulate PHB when grown
under dinitrogen-fixing conditions. There can be a wide
variation in PHB content between species and between strains
of the same species (Stockdale et al., 1968). The regula-
tion of PHB levels in Azotobacter beijerinckii has been
extensively studied and may provide clues to the role of PHB
in the physiology of other free-living, dinitrogen-fixing
bacteria, such as azospirilla.

The route of PHB biosynthesis in A. beijerinckii has
been reviewed by Dawes (1981). The synthesis and degrada-
tion of PHB in this microorganism are intimately associated

with intermediates and enzymes of the tricarboxylic acid (TCA) cycle, a system that azospirilla also possess (Okon et al., 1976b). When A. beijerinckii strain N.C.I.B. 9067 was cultured as a dinitrogen-fixer with 2.0% (wt/vol) glucose, PHB was deposited towards the end of exponential growth. The cells were unable to use all the available glucose, and PHB synthesis continued during the stationary phase until up to 74% of cell dry weight was PHB. Cultures grown with combined nitrogen rarely contained more than 3.0% of their dry weight as PHB (Dawes, 1981).

The initiation of PHB synthesis in the A. beijerinckii strain in batch culture coincided with the attainment of zero-oxygen concentration. Oxygen limitation was thus suspected to be a critical factor in initiating PHB synthesis. However, the nature of batch broth culture made it hard to separate oxygen effects from possible nitrogen-limitation effects (Senior and Dawes, 1971). Later experiments, using chemostat cultures having carbon, oxygen, or nitrogen limitation, clearly showed that extensive PHB accumulation only occurred under conditions of oxygen limitation (Dawes, 1981).

Before the studies reviewed by Dawes (1981), PHB was regarded as being only an endogenous, carbon-and-energy source that benefited cells during starvation. These experiments suggested that PHB could also serve other purposes. The synthesis of PHB seemed to serve as an

[illegible] (NADH and NADPH) that accumulated and the cell became oxygen limited, and electron transport to oxygen via the terminal oxidases of the electron-transport chain was restricted (Senior and Dawes, 1971). Later work revealed that the activities of certain enzymes of carbon catabolism in *A. beijerinckii* are inhibited by either or both NADH and NADPH. Under oxygen limitation, the concentration of these reduced coenzymes is increased, so that glucose metabolism, operation of the TCA cycle, and net biosynthesis are decreased. Growth can continue at some level, however, if PHB is synthesized and the crucial coenzymes are reoxidized (Dawes, 1981).

The synthesis of PHB under oxygen limitation may occur in other bacteria as well (Okon and Hardy, 1983). The quantity of PHB accumulated often greatly increases as the C/N ratio of the growth medium increases. Under such conditions, free-living dinitrogen-fixers may assimilate the exogenous carbon more rapidly than they can produce reduced nitrogen. As a result, the cells can accumulate large amounts of PHB (Stevenson and Socolofsky, 1966; Dawes and Senior, 1973). The metabolism of PHB is regulated such that PHB accumulates when the supply of exogenous carbon is in excess of the requirements for growth and maintenance, and it is degraded when the supply of exogenous carbon is limited or exhausted (Dawes, 1981), or when balanced growth can again occur (Nickels et al., 1979).

[redacted] shown that PHB can accumulate [redacted] ro- growing or proceeding toward cell division, [redacted] limitation of available nutrients (Dawes and Senior, [redacted] Nickels et al. (1979) demonstrated this in laboratory m[redacted] cosms containing oak leaf detritus and estuarine water. Supplementing the nutrients in the water column with carbohydrates, especially glucose, induced a rapid accumulation of PHB without a concomitant increase in microbial biomass. When supplements were added that enabled increases in microbial biomass, PHB levels fell as the polymer was broken down to aid microbial growth.

In one study, A. brasilense Sp 7 was grown in batch cultures for up to 14 days in microaerophilic, nitrogen-free malate broth (Pepen and Werner, 1980). Both nitrogenase activity and PHB synthesis were biphasic. An initial peak of PHB content occurred at day 3, 1 day before the first peak of nitrogenase activity. During the first and maximal peak of nitrogenase activity, there was a decrease in PHB content, possibly due to accumulation of fixed nitrogen allowing use of PHB carbon skeletons for biosynthesis. A second peak of PHB accumulation occurred after the first maximum of nitrogenase activity. The results suggested that A. brasilense Sp 7, like A. beijerinckii, may accumulate PHB when it assimilates exogenous carbon faster than it can fix dinitrogen.

Zimmer et al (1984) found [illegible] accumulated PHB when using nitrite as terminal electron acceptor for anaerobic growth. A maximum of 38% of cell dry weight was found to be PHB when less than 3.0 mM nitrite was present. No PHB was accumulated when in excess of 8.0 mM nitrite was made available, indicating the role of PHB as a sink for excess reducing power when other electron acceptors are scarce. It was also found that PHB-rich cells contained less protein than did PHB-poor cells.

Azospirillum lipoferum strain Sp 17 (ATCC 29709) was found by Volpon et al (1981) to accumulate nearly 24% of its dry weight as PHB near the mid-logarithmic phase of growth as a dinitrogen-fixer. Near the end of logarithmic growth, PHB synthesis seemed to stop, and the content of PHB declined to 13% of cell dry weight in stationary phase.

The PHB metabolism of A. brasilense strain Cd (ATCC 29729) has received considerable study. When this strain was grown in continuous chemostat culture with malate and ammonium chloride, a maximum PHB content of 12% of the biomass was observed under microaerophilic conditions and at intermediate growth rates (Nur et al., 1982). These growth conditions were said to approximate conditions generally encountered in the rhizosphere. The production of PHB was markedly decreased at higher levels of oxygen and higher growth rates. Once again, it was observed that cells

containing high [illegible] than PHB-poor cells.

Recent work by Tal and Okon (1985) has further delineated the roles PHB may play in the physiology of A. brasilense strain Cd. Grown in aerobic batch culture with malate and 2.8 mM NH_4Cl, the cells accumulated 40% of their dry weight as PHB after 24 hours, toward the end of exponential growth. When the level of NH_4Cl was raised to 15.0 mM, the cells accumulated only 5% of their dry weight as PHB after 24 hours. In both cases, the amount of PHB decreased in stationary phase.

In chemostat continuous culture, a maximum of 30% cell dry weight accumulated as PHB when the gas atmosphere was 0.082 mM O_2 (Tal and Okon, 1985). With increasing aeration, the PHB content fell to very low levels. When grown in batch culture as dinitrogen-fixers, the cells accumulated about 75% of their dry weight as PHB. Maximal PHB content was obtained in these experiments when the C/N ratio was about 70. Both the C/N ratio of the medium and the oxygen concentration were found to regulate PHB synthesis.

The forms of carbon and nitrogen made available to the cells affected the levels of PHB accumulated (Tal and Okon, 1985). Organic acids, especially pyruvate, were found to elicit PHB formation more than carbohydrates did. Sodium nitrate was found to promote PHB formation more than ammonium chloride did, possibly because nitrate does not

accumulate [redacted] ammonium.

Cells with different contents of PHB were [redacted] by centrifugation and resuspended in phosphate buffer to measure viability during aerobic nutrient starvation. By 140 hours, bacteria with abundant PHB reserves had given rise to more than twice as many viable cells as were present in the initial inoculum (Tal and Okon, 1985). During starvation, PHB reserves were degraded quickly but not completely. The initial inoculum contained 40% of its dry weight as PHB. This fell rapidly to about 24% of cell dry weight after 42 hours of starvation. After 130 hours of starvation, the PHB content of the cells was about 20% of cell dry weight.

In comparison, cells initially containing only 5% of their dry weight as PHB had only 7% of the original number of viable cells after 130 hours of starvation (Tal and Okon, 1985). Poly-β-hydroxybutyrate was still measurable throughout starvation of these PHB-poor cells, stabilizing at or near 3% of the dry weight of all cells present.

Starved PHB-rich cells had a higher respiration rate during starvation than the PHB-poor cells (Tal and Okon, 1985). Unlike cells having low amounts of PHB, the PHB-rich cells exhibited nitrogenase activity in the absence of exogenous carbon sources. But the PHB-rich cells were as unable to reduce nitrate anaerobically as were the PHB-poor cells in the absence of exogenous carbon.

This study (Tal and Okon, 1985) also suggested that elevated PHB levels at the onset of starvation may spare the use of protein to drive endogenous metabolism. The PHB-poor cells used up two-thirds of their initial protein during the first 80 hours of starvation, whereas the protein content of starved PHB-rich cells increased slightly over 80 hours. It was also reported that PHB-rich cells were able to survive a variety of environmental stresses, including desiccation, better than PHB-poor cells (Tal and Okon, 1985).

The previous study also found that cells enriched in PHB displayed a one hundred-fold higher aerotactic response than PHB-poor cells. This supports the claim made in an earlier study that PHB reserves could be used for aerotaxis when no exogenous carbon source was available (Barak et al., 1982).

The previous discussion has shown that both capsule and PHB synthesis can be promoted by environments with high available C/N ratios. The roles of capsules and PHB in pleomorphism in azospirilla will be discussed later. The nature of dormancy in prokaryotic cells will be discussed first, since some pleomorphic forms of azospirilla may be dormant stages. Capsular layers and PHB are often present in dormant forms of prokaryotes.

Dormant Forms of Prokaryo[illegible]

There is general agreement that mo[illegible] spend much of their existence in soil in a state of low metabolic activity. The low respiratory rates of bulk samples of nonamended soil support this (Clark, 1967). Many soil bacteria may be metabolically dormant due to a lack of readily available carbon and energy supplies (Gray and Williams, 1971). Soil bacteria may enter into exogenous dormancy, where growth is delayed by unfavorable physical or chemical conditions (Marshall, 1980). Such bacteria probably have the same morphology as actively growing vegetative cells (Gray and Williams, 1971). These cells are probably intimately associated with the clay or organic matter of soil. The cells adsorb to these surfaces by physical or chemical interactions, or by the use of exopolysaccharides (Stotzky, 1980).

However, many bacteria may exist in soil as dormant forms that are morphologically different from their growing, or vegetative, stages. These cells would have entered a phase of constitutive dormancy, involving the formation of spores or cysts (Marshall, 1980). Bae et al. (1972) used transmission electron microscopy to study thin sections of bacteria released from soil by centrifugation and washing. About 28% of the bacteria observed had normal vegetative morphology, of which 29% possessed capsular layers.

[illegible] comprised [illegible] observed

Sudo and Dworkin (1973) reviewed the kinds of prokeryotic resting cells that were recognized at the time. Bacterial resting cells were defined as cells in which division does not occur, and endogenous respiration is absent or greatly reduced. Usually resting cells are more resistant to environmental stresses than are vegetative cells. Resting cells are often morphologically different as well. Such resting cells often differ in chemical composition from vegetative cells (Keynan, 1972). There are often either qualitative or quantitative differences between the electron transport systems of vegetative and resting cells. Many resting cells, for some period after they have germinated and resumed growth, are self-sufficient in energy sources, metabolites, and macromolecular precursors.

Perhaps the best understood bacterial resting cell stages are the endospores of bacilli and clostridia. Cysts differ from endospores in that they are formed by the modification of an entire vegetative cell. The vegetative cell rounds up during encystment and becomes coated with one or more layers, often exopolysaccharide, external to its cell wall. No cyst forms withstand the extremely high temperatures tolerated by endospores, but they are comparably resistant to other environmental stresses (Sudo and Dworkin, 1973).

Certain properti[illegible] of prokaryotes. They are formed when the growth rate of vegetative cells declines (a metabolic shift-down), due either to nutrient depletion or transfer of cells to an environment where balanced growth can no longer occur. Cells encountering these conditions complete their ongoing synthesis of DNA and chromosome replication but do not initiate new rounds of DNA synthesis, since growth has ceased (Sadoff, 1975). Conditions that will prohibit further growth promote the formation of dormant cells that can survive stress better than vegetative cells. These dormant cells often contain PHB or other energy-reserve polymers, and thickened cell walls or capsular layers. They have enhanced resistance to irradiation, sonic vibration, and sometimes elevated temperatures. Perhaps the most important traits for survival of dormant cells in natural environments are their resistance to starvation, low endogenous respiration rates, and desiccation resistance. Many cells entering constitutive dormancy need time to mature before they achieve maximal resistance to stress. It is important to remember that resting cells formed in natural environments may differ qualitatively and/or quantitatively in their resistance properties from those formed under laboratory conditions (Sudo and Dworkin, 1973).

Many strains of Gram-negative myxobacteria form dormant cells, called microcysts, when nutrients become limiting

Nonmotile, encapsulated, mature microcysts are more resistant than are vegetative cells to environmental stresses such as ultraviolet irradiation, sonic vibration, and desiccation (Sudo and Dworkin, 1973).

Many Gram-negative, methane-oxidizing bacteria isolated from soil or mud are encapsulated and accumulate PHB when nitrogen becomes limiting for growth (Whittenbury et al., 1970b). Depending on the genus and strain, up to 90% of the cells present may form resting cells upon entering the stationary phase of growth. Lipid cysts of Methylocystis parvus accumulate large amounts of PHB and survive starvation and desiccation better than vegetative cells, but lack well-defined, capsular cyst coats. Methylomonas spp. and Methylococcus spp. form rounded, nonmotile cells that survive starvation better than do vegetative cells. These cells are called immature cysts, because they never attain desiccation resistance. Some strains of Methylobacter spp. form starvation-resistant and desiccation-resistant cysts that seem morphologically identical to Azotobacter spp. cysts (Whittenbury et al., 1970a).

Bdellovibrio sp. strain W is the only bdellovibrio that is known to encyst. Bdellocysts are larger than their vegetative counterparts and are not light-refractile. They tolerate sonic disruption, ultraviolet irradiation, and carbon starvation better than do vegetative cells. The endogenous respiration rate of bdellocysts is 80% less than

that of vegetative cells. When dried over silica gel desiccant under slight vacuum in glass tubes, vegetative cells of strain W die out rapidly and entirely. From 45% to 80% of Bdellocysts initially present are able to survive 6 days of this desiccation treatment (Tudor and Conti, 1977). Bdellocysts possess a thickened outer layer of modified peptidoglycan, and contain inclusion bodies of an amylopectin-like polysaccharide of glucose monomers. These features are not found in vegetative cells (Tudor, 1980).

Some strains of Azotobacter spp., apparently some azospirilla, and the bacteria described above are the only prokaryotes reported to form cysts. Why do not more bacteria possess resting stages that are morphologically differentiated into cysts? Perhaps growth media and conditions used in the laboratory discourage cyst formation (Whittenbury et al., 1970b). It is also possible that the ability to form cysts is sometimes labile and may be lost upon subculture. One Methylobacter chroococcum strain was able to form multiple-bodied cysts upon initial isolation from the environment. It ceased to do so when subcultured. Other Methylobacter spp. have retained the ability to form single- and multiple-bodied cysts over several years of subculture (Whittenbury et al., 1970b).

A mature, cyst-like cell of a prokaryote may perhaps best be characterized as follows. Mature cysts differ morphologically from vegetative cells in having thickened

[illegible]. They are [illegible] have [illegible] endogenous [illegible] rates. They only initiate growth into vegetative [illegible] when sufficient nutrients are available. They [illegible] also possess more resistance to some environmental stresses than do vegetative cells. Enhanced resistances to starvation and desiccation are probably traits of all mature cysts. The cysts of the methane-oxidizing bacteria and of Azotobacter spp. possess these characteristics. Mature cysts of azospirilla should also have these properties.

Desiccation resistance is a critical characteristic of prokaryotic cysts. The next section will consider experiments conducted to assess the resistance of bacteria to drying.

Resistance of Bacteria to Drying

Clark (1967) stated that the majority of soil bacteria survive in air-dried soils, often for several years. When such soils are rewetted, bacterial activities including nitrification, ammonification, nonsymbiotic dinitrogen fixation, and sulfur oxidation are usually detected. The implication is that the intimate association of bacteria with clay or organic matter allows bacterial survival at hydrated microsites in a macroscopically dry soil. Later findings, reviewed by Stotzky (1980) and Marshall (1980), support this. Exopolysaccharides may help bacteria to achieve such intimate association, although capsules

themselves have not been fo[illegible] or resistance in laboratory studies [illegible] nonsoil conditions (Dudman, [illegible]

Because of the importance of desiccation as a limiting factor in legume inoculation with Rhizobium spp , several studies have been done on their resistance to drying. There are broad strain differences in resistance of rhizobia to desiccation. Many variables are present in drying experiments, and the variables may interact with one another. Rhizobium spp. withstand drying best in heavier-textured soils, where hygroscopic water can be retained by colloidal surfaces. Die off is far more rapid in drying sand. Capsules do not afford increased resistance to drying in studies with soil or other drying surfaces (Lowendorf, 1980). Often fewer rhizobia survive rapid drying procedures, such as oven drying, than survive milder desiccation over several weeks' time with controlled relative humidities (Jansen van Rensburg and Strijdom, 1980).

Robinson et al. (1965) added pure cultures of Pseudomonas spp. or Arthrobacter spp. to sterile soils. The inoculated soils were dried by passing filtered air through them for 2 days, by which time they had reached constant weight. This forced drying resulted in rapid die off for both species. Labeda et al. (1976) found that slow evaporative drying of inoculated soil resulted in reduced death rates for both Pseudomonas spp. and Arthrobacter spp.

[illegible] show clearly that rate of [illegible] can profoundly affect the survival of vegetative bacteria. Vegetative cells with the capacity to become desiccation resistant may need time to alter their membrane or cytoplasmic composition before desiccation resistance is achieved. Fast-drying procedures may not allow them to do so. A differentiated resting cell, such as a cyst, may also need time, depending on how mature it is, to become desiccation resistant.

Relative humidity (RH) also has a great influence on desiccation resistance of prokaryotes. In desiccation at any RH below 90%, the free water of the cells is removed almost instantaneously. The water that remains is the bound water content of the cell, which may be necessary for continued function of essential metabolic processes and viability. Often few vegetative bacteria die when desiccated above 70% RH, but die rapidly as the RH declines to 45% (Webb, 1965). Many desiccation studies have not defined the RH at which the cells were dried, making duplication of results difficult.

Thompson and Skerman (1979) tested the desiccation resistance of vegetative cells of many strains and genera of the Azotobacteraceae. One milliliter samples of vegetative cell cultures were added to sterile porcelain beads, positioned above silica gel in glass bottles sealed with Parafilm. These desiccation units were stored at room

temperature and at different times single beads were aseptically removed and placed in broth media. The bacteria were probably in stationary phase when added to the assemblies, but it is unlikely that many cysts were present even in stationary phase broth culture (Sadoff et al., 1971). The results were surprising: the majority of strains retained viability for 1 to 2 years of desiccation. This was true even for bacteria that have never been shown to form cysts.

Mature cysts of prokaryotes survive rapid desiccation on glass surfaces far better than do their vegetative counterparts, but rarely does all the encysted inoculum survive rapid drying. Cysts of methane-oxidizers retained 60% to 90% viability after 1 week (Whittenbury et al., 1970a), and Sphaerocysts retained 45% to 80% viability after 6 days (Tudor and Postgate, 1977). This may mean that not all the encysted cells were fully mature when exposed to drying, even if they all appeared morphologically identical. Such quick-drying assays can be valuable in determining whether morphologically differentiated cells are truly cyst-like.

Differences in the desiccation resistance of Azotobacter spp. vegetative cells and cysts are usually determined by the method of Socolofsky and Wyss (1962). They impinged suspensions of either cell form on the surfaces of membrane filters. The filters were then transferred to dry adsorbent pads in Petri dishes and placed in

an incubator at 33°C. This method is a slow-dying procedure. At different time intervals, the cells were washed from the membranes, and viability was determined by plating. Cysts of Azotobacter vinelandii ATCC 12837 lost little viability over a 12-day period using this drying treatment, whereas 95% of the vegetative cells were killed by the end of the first day (Socolofsky and Wyss, 1962). As a result of the rapid die off of vegetative cells with this treatment, later studies considered cells of this strain to be cysts if they could withstand 4 days of desiccation on membrane filters (Stevenson and Socolofsky, 1966; Wyss et al., 1969).

None of these membrane filter studies specified the RH at which the membranes were dried, or how many cell layers were deposited upon the membranes. Webb (1965) pointed out that if bacteria are dried on filters to test their desiccation resistance, the cells must be applied in a monolayer to achieve consistent results. If more than a cell monolayer is on the filter, most of the cells in subsurface layers will not be dried or equilibrated with the water vapor of the environment.

Most desiccation resistance experiments have given ill-defined or incomplete conditions of drying. Such experiments have proven, however, that cysts are more desiccation resistent than are their vegetative counterparts

Vela (1974) tested desiccation resistance of Azotobacter vinelandii ATCC 12837 by allowing slow [illegible] the agar on which the cells were grown. Vegetative cells were grown on agar plates of Burk's nitrogen-free medium, with glucose as the carbon source. Cysts were obtained by growing the cells on the same agar, except that 0.3% (vol/vol) n-butanol was employed as sole carbon source. Dried agar films were then broken with sterile forceps and placed on the surface of Burk's agar medium containing glucose. Vegetative cells borne on these agar films remained viable for nearly 2 years of desiccation, whereas cysts borne on such films remained viable for 10 years or longer.

Desiccation tolerance of azospirilla has received some attention. Lakshmi et al. (1977) recovered azospirilla from several air-dried soils stored in the laboratory. Recovery was obtained from one of four sandy soils stored air-dry for 10 years. All of these soils had less than 0.5% organic matter. Heavier-textured soils with 1.0% or more organic matter consistently yielded isolates of azospirilla. Some of these heavier-textured soils had been stored air-dry for up to 15 years. It was suggested that organic matter aids the survival of azospirilla in drying soils, and that desiccation-resistant cells may be formed by these bacteria.

Jagnow (1982) did some work with an Azospirillum lipoferum strain isolated from maize roots. In field

[illegible] CFU/g soil, azospirilla [illegible] on roots survived better than those in soil distant from roots. When added to pots of soil containing grass and cereal plants, populations remained at 10^6 to 10^7 CFU/g soil, even after 70 days of drought. He speculated that the presence of roots, either living or dead, enhances the drought tolerance of the associated azospirilla. In laboratory studies using nonautoclaved soil microcosms, air drying of soil was found to kill greater than 99% of the initial Azospirillum lipoferum inoculum. In comparison, the indigenous bacteria were little affected by air drying. This perhaps indicates that, unless azospirilla added to soil are able to associate quickly with plant roots, they will soon die out if drought stress occurs.

The desiccation resistance of pleomorphic encapsulated forms of azospirilla has been studied. Lamm and Neyra (1981) studied A. brasilense Sp 7 and A. lipoferum Sp 59b, in addition to several strains of azospirilla isolated from roots of various grasses in New Jersey and New York. To obtain cyst-enriched cultures, cells grown in nutrient broth were harvested by centrifugation, then washed and resuspended in sterile 0.85% (wt/vol) NaCl. A 1.0 ml sample of cells was then spread plated as a lawn onto nutrient agar plates containing 2.0% (wt/vol) agar. Plates were incubated at 30°C until the agar was dried into a thin film, often requiring a month. After 15 days of incubation, cyst-like

[illegible] Photographs of [illegible]ched cultures showed that many vegetative cells were still present. To obtain cyst-free cultures, cells were grown in nutrient broth, then washed and resuspended in saline. These cells were then spotted onto sterile, predried nutrient agar films so that the added cells would dry completely on the agar film in 30 min at 30°C. Agar films from each treatment were then cut with sterile scissors and aseptically transferred to vials containing silica gel. To test viability, the dried agar films were removed periodically from the vials, placed on nutrient agar plates, and incubated for 1 week at 30°C. Vegetative cells did not survive the initial drying process. Cyst-enriched populations that survived the initial desiccation period remained viable for up to 15 months. Interestingly, cyst-enriched cultures of two root isolates were nonviable at time zero, when they were placed into the silica-gel vials (Lamm and Neyra, 1981)

Two aspects of this study deserve special comment. Clearly, the cyst-enriched cultures did not receive the same drying treatment as did the vegetative cells. The cyst-enriched agar films were obtained by a slow drying process, and the vegetative cell agar films underwent rapid drying. It does not seem valid to compare their desiccation tolerance under these different conditions. Also, two strains that contained cyst-like cells of apparently mature morphology were not desiccation resistant. Perhaps they

[illegible] experimental conditions

Papeu and Werner (1982) assessed the desiccation resistance of cyst-like forms of A. brasilense Sp 7. Cell from dinitrogen-fixing broth cultures were diluted in sterile tap water and then impinged onto the surface of sterile 0.2 µm Millipore® membrane filters under vacuum. Some of the filters were immediately placed on the surface of nutrient agar plates and incubated at 28°C, whereas others were placed on sterile adsorbent pads in Petri dishes and dried at 37°C until they were placed on nutrient agar plates. Desiccation-resistant cells were only present after the first peak of nitrogenase activity, when nonmotile, encapsulated spheres containing PHB predominated. Cells before and during the first peak of nitrogenase activity were motile vibrioids and did not survive the desiccation treatment. As a second peak of nitrogenase activity arose, motile, dinitrogen-fixing vibrioids emerged from the spherical capsules, these vegetative cells were again not desiccation resistant. More encapsulated, spherical cells survived 2 days of desiccation than 6 days, but it was not an order of magnitude difference. This again may be an indication that morphologically mature cysts are not necessarily physiologically mature.

The recent work of Sadasivan and Neyra (1985) employed another assay for desiccation resistance of cyst-like forms

of azospirilla. Azospirillum brasilense Sp 7 and A. lipoferum Sp 59b were studied. Large flocs of cells enclosed in exopolysaccharide were placed on Whatman® No. 1 filter paper and air-dried for 30 min. They were then placed in a closed vial, without desiccant, and incubated at 30°C for up to 6 months. Small pieces of dried flocs were transferred periodically to semisolid nitrogen-free malate medium and incubated at 34°C for 2 to 4 days, and growth, pellicle formation, and nitrogenase activity were observed. Cells in dried flocs remained viable for up to 6 months of drying.

No vegetative cell controls were dried and tested for viability in the above study. Although cells remained viable in dried flocs for up to 6 months, it is not known how many cells survived in a given amount of floc. It is not known whether the cells themselves were desiccation resistant, or only physically protected from desiccation by exopolysaccharides.

Tal and Okon (1985) claimed that PHB-rich cells of A. brasilense strain Cd were 10 times more desiccation resistant than cells having little of the polymer. No details of the test used for desiccation resistance were given.

Desiccation resistance studies can be difficult to interpret. Comparing the desiccation resistance of vegetative cells to that of cysts may be less difficult than

comparing that of vegetative cells of different s[illegible]
Rapid drying on a glass surface should enable most m[illegible]
cysts to remain viable, but not most vegetative cells [illegible]
glass drying surface should be less hygroscopic than are membrane filters or agar films. Rapid drying on a glass surface is a severe treatment, but it should reveal the presence of physiologically modified, stress-resistant cells, such as mature cysts.

Azotobacter Cysts

Discussion of the nature of Azotobacter spp. cysts is important, because this information served as the basis for the experiments with azospirilla reported in this study.

Like azospirilla, the Azotobacteraceae are Gram-negative aerobes, often containing PHB granules. Many are motile by flagella. They all fix dinitrogen, and some, including Azotobacter spp., do so either at atmospheric oxygen levels (unlike azospirilla), or as microaerophiles. Only one genus, Azotobacter, contains species with strains that are known to form cysts (Tchan, 1984). The isolation of Azotobacter spp. from the interior of 2,000-year-old clay bricks (Abd-El-Malak and Ishac, 1966), and their persistence in soils that had been air dried from 10 years (Vela, 1974) to 30 years (Clark, 1967), may be due largely to cyst formation.

When grown in nitrogen-free broth with glucose as the carbon source, young cells of Azotobacter spp. appear as rods with rounded ends, ranging from 1.3 to 2.7 μm in diameter and 3.0 to 7.0 μm in length. As cultures age, cells often accumulate PHB. Cell morphology may be altered to ellipsoids, filamentous cells, or chains of cells (Tchan, 1984).

Azotobacter spp. are commonly isolated from soil and aquatic habitats of near-neutral pH, and are generally less acid-tolerant than azospirilla. The most common species isolated from soil is Azotobacter chroococcum, but its biochemistry and physiology have received less attention than that of Azotobacter vinelandii (Tchan, 1984). Azotobacter vinelandii ATCC 12837 forms cysts profusely under appropriate growth conditions. When this strain is cultured in Burk's nitrogen-free broth with glucose, some cysts form in stationary phase cultures, but only 1.0% (Lin and Sadoff, 1969) to 10.0% (Reusch and Sadoff, 1981) of the population encysts under these conditions.

Early workers such as Winogradsky (1938) knew that growing some Azotobacter spp. in nitrogen-free media, with ethanol or butanol as carbon source, led to enhanced production of nonmotile, spherical cells with double-layered coats. Socolofsky and Wyss (1961) built upon this knowledge, using A. vinelandii ATCC 12837 (which was used in all the studies that follow unless otherwise indicated).

When cultured as cell lawns on Burk's nitrogen-free agar with 0.3% (vol/vol) n-butanol as sole carbon source, cysts began to appear within 3 days and predominated in 5 to 7 days. Ultrastructural studies revealed that the outermost layer of the cyst, the exine, consisted of several overlapping, plate-like layers. Beneath the exine was a much thicker layer of gelatinous material, called the intine. The intine surrounded a modified resting cell, called the central body, which often contained numerous PHB granules. Cysts had no detectable endogenous respiration when suspended in buffer, but almost instantaneously began measurable respiration when exogenous carbon sources were added. In later studies, cysts were produced by growth on 0.2% (vol/vol) n-butanol (Socolofsky and Wyss, 1962), or 0.2% (wt/vol) β-hydroxybutyrate (BHB) (Lin and Sadoff, 1968), with 90% or greater of the cells being converted to cysts in 5 to 7 days.

Eklund et al. (1966) demonstrated that the formation of capsular layers by vegetative cells was a prerequisite for cyst formation. Complete morphological encystment of cells grown on n-butanol agar with various levels of NH_4NO_3 only occurred in the usual 5-day-period when the NH_4NO_3 concentration was 0.02 M or less. The cells rounded up within 5 days into nonmotile precysts lacking exines when 0.03 M or 0.04 M NH_4NO_3 was initially present. By day 10, these cells had used up enough of the original combined nitrogen to

allow nitrogen fixation to resume so that capsular polysaccharide was produced, followed by formation of exine and, ultimately, morphologically mature cysts. Nonencapsulated mutants were unable to form morphologically mature cysts. The work of Pope and Wyss (1970) emphasized that cells beginning encystment first produced a capsule that acted as a structure within which the cyst coats were built, so that the exine existed inside of the capsule. The diameter of morphologically mature Azotobacter cysts measured between exine boundaries is about 2.0 µm (Reusch and Sadoff, 1983).

Abortive encystment occurs when cells round up into nonmotile precysts, but are unable to form a complete exine. This occurs in the presence of high amounts of combined nitrogen (Eklund et al., 1966), when glucose or other carbon sources are present in addition to n-butanol or BHB (Lin and Sadoff, 1968), or when calcium is unavailable. The calcium requirement is probably related to its function as a stabilizing cation that holds the cyst coats together (Page and Sadoff, 1975). Using 3.0 mM EDTA in 0.05 M Tris buffer, pH 7.8, Lin and Sadoff (1969) obtained almost instantaneous expulsion of the central body from the cyst coats, due to the chelating effect of the buffer. The empty exines had the same "horseshoe" shape seen when cysts germinate, and vegetative cells separate from the exines.

The role of PHB in cyst formation was examined by Stevenson and Socolofsky (1966). Cysts were defined as cells that could survive desiccation on a membrane-filter surface for 4 days at 33°C. After 2 days of growth on nitrogen-free n-butanol agar, cells lost their motility, became oval-shaped, and accumulated PHB to the extent of 55% of cell dry weight. The development of mature cysts was accompanied by a reduction in PHB content. By 6 days, cultures had undergone 100% encystment, and 10% of cyst dry weight was PHB.

Lin and Sadoff (1968) developed a two-step replacement procedure for obtaining cysts in broth. Cells were grown to late exponential phase in Burk's nitrogen-free broth with glucose. After harvest by centrifugation and washing in buffer, cells were resuspended in Burk's salts broth with 0.2% (wt/vol) BMB. This procedure was used in further studies (Hitchins and Sadoff, 1970, 1973; Reusch and Sadoff, 1979, Su et al., 1981; Rausch and Sadoff, 1983), resulting in the following detailed description of BMB-induced encystment.

After 1 hour in encystment broth, cells are still motile and flagellated but no longer possess nitrogenase activity. Within 4 to 6 hours, DNA synthesis has ceased, and soon afterward each cell divides to form two nonmotile precysts. There is rapid accumulation of PHB during this period, and the rate of phospholipid synthesis declines.

Simultaneously, BHB is being taken up and respired or incorporated. From the sixth to sixtieth hour, unique lipids, not found in vegetative cells, begin to be produced. These include 5-n-alkylresorcinols (AR1) and their galactoside derivatives (AR2). These lipids possess hydrophobic alkyl sidechains and hydrophilic phenolic heads. Also produced are 6-n-alkylpyrones (AP), having a similar bipolar nature. During this time, membranous vesicles migrate outward from the central body through the intine to form the exine layer. Up to 17% of the exine is composed of AR1 and AR2. The central body produces AR1 and AR2 in part from its PHB reserves, and exports them in the membranous vesicles to the exine region. Radio-labelled BHB accumulates in the central body and exine, whereas the intine contains almost none. This indicates that the intine is composed mainly of capsular material, formed from cell reserves that were present before encystment is triggered by BHB. Net RNA synthesis stops by the twelfth hour, and net protein synthesis continues for up to 36 hours. Lipid turnover continues beyond 60 hours, but there is no net lipid synthesis. In a mature cyst, 5.0% of the central body membranes are phospholipid, with AR and AP composing the other 95%. Molecular models suggest that AR and AP form a more rigid membrane structure at physiological temperatures than do phospholipids. The hydrophobic, viscous nature of

such a membrane may contribute greatly to the desiccation resistance and dormancy of cysts.

The possible contribution of the central body membranes to stress resistance of cysts was suggested in earlier studies. The cysts of Azotobacter chroococcum strain 75-I had a compact, well-defined exine layer, whereas the exine of A. chroococcum strain NTS was diffuse and fragile (Vela and Cagle, 1969). The cysts of A. chroococcum strain 75-I were much more resistant to sonic disruption than cysts of A. chroococcum strain NTS. Yet cysts of both strains were comparably resistant to desiccation on membrane filters and to ultraviolet irradiation. Kramer and Socolofsky (1970) defined cyst germination of A. vinelandii ATCC 12837 as a process whereby desiccation resistance is lost; mature cysts were defined as cell forms surviving 3 days of desiccation on membrane filters. It was found that 40.0 µg chloramphenicol/ml inhibited outgrowth of cysts in a complete medium. Many cysts lost their desiccation resistance when incubated with chloramphenicol, indicating that the antibiotic might have chemically changed some essential cyst component, perhaps the central body's membranes. Hitchins and Sadoff (1973) found that, soon after exposure to EHS, vegetative cells became resistant to 100.0 µg chloramphenicol/ml. The antibiotic had no affect on morphogenesis or rates of protein synthesis. This is another indication of rapid membrane alteration of encysting

[illegible] lon, before AR and AP are produced. Further support for the importance of membranes may be found in studies where mineral nutrient deficiencies lead to the production of stress-resistant cysts which lack completed cyst coats (Gonzalez-Lopez et al., 1985).

Germination of cysts has usually been defined as the emergence of a growing, motile cell from the exine layer (Socolofsky and Wyss, 1961). Loperfido and Sadoff (1973) examined the germination of cysts exposed to glucose. Cysts respired detectably within 2 min after the addition of 1.0% (wt/vol) glucose, and soon afterwards net synthesis of RNA and protein became measurable. After 4 to 6 hours, the central body had enlarged to occupy the volume of the intine, and DNA synthesis and nitrogenase activity became measurable. After 8 hours, a vegetative cell emerged from the cyst coats, leaving behind an empty "horseshoe"-shaped exine. Germination did not occur in the absence of oxygen. Cysts also germinated in the presence of sugars other than glucose. Germination did not occur in Burk's nitrogen-free salts, indicating that the PHB reserves of the cysts could not be mobilized to initiate germination. The addition of 0.25% (wt/vol) NH_4^+ did not lead to germination.

When cysts are germinated on glucose, some central bodies divide within their cyst coats to form multiple central bodies. Up to six central bodies have been observed within one cyst coat (Cagle and Vela, 1974).

Pleomorphism of Azospirill

Bacteria cultured in vitro can be extr[illegible]l, pleomorphic. Only a few cells in a population may exhib[illegible] abnormal morphology under some cultural conditions, but sometimes the majority of a culture assumes unusual shapes. Older cultures in the stationary growth phase can be especially pleomorphic (Duguid and Wilkinson, 1961).

Hughes (1956) has reviewed the development of bacterial filaments. Filamentous cells are usually as wide as normal cells, but are several times longer and lack developed septa. They are interesting because they are often fully viable, unlike some pleomorphic or involution forms of bacteria. Under suitable cultural conditions, a filament may divide at several points along its length to produce several cells of normal length. Filaments can be induced by sublethal cell damage, interruption of balanced growth, dyes and antibiotics, extremes of pH, refrigeration, and various forms of radiation.

Slater and Schaechter (1974) emphasized how sensitive bacterial cell division is to the factors mentioned above. If sublethally stressed, rod-shaped bacteria may continue to grow and form filaments. Filaments can also form during very rapid growth in rich media, and will fragment into individual cells when growth slows, or when the environment becomes less nutritionally rich. Since cells arising from fragmentation of filaments are usually of normal length, the

cell's ability to control the site of cell divi[illegible] is lost during filamentation. Sometimes chains of cells occur instead of filaments. The cells in chains contain septa, but final cleavage between cells has not yet occurred. It is possible that contiguous cells having incomplete septa in such chains share continuities between their cytoplasms. In some cases, chains may be held together by very thin capsular layers common to several cells in the chain.

Jensen and Woolfolk (1985) found that several strains of Pseudomonas putida and Pseudomonas fluorescens were induced to form filaments if oxygen became limiting during the late logarithmic phase of growth in nutrient broth. Exhaustion of one or more nutrients was also a probable elicitor of filamentation. The weakly motile filaments, unlike the highly motile aerobic rods of the bacteria, migrated to microaerophilic zones. As respiration of cultures declined, the increasing levels of oxygen in the broth seemed to trigger fragmentation of the filaments into rods. Cultures containing filaments, or the progeny of fragmented filaments, retained viability longer than nonfilamentous cultures.

Morphological changes in Escherichia coli have been related to specific genes. If cellular DNA is damaged by ultraviolet irradiation or other influences, several genes are expressed in the so called SOS response. Many of the

[illegible] others specifically block [illegible] cell [illegible] the DNA is repaired, cell division is blocked, but cells can continue to grow into long, nonseptate filaments. Upon repair of the DNA, septa form along the filaments, and cells of normal size are produced after septum separation (Donachie et al., 1984). Certain E. coli mutants are known to produce septa, but form chains because the enzymes needed for septum cleavage are not produced (Begg and Donachie, 1985).

Thompson and Skerman (1979) showed that most members of the Azotobacteraceae are pleomorphic under certain cultural conditions. Filaments and chains of cells are produced commonly. Similar pleomorphism has been observed with azospirilla.

Becking (1982) observed that the morphology of azospirilla varied in different culture media. On yeast extract-glucose agar, the cells were highly motile, slightly curved rods, 2.0 to 4.0 µm long and 1.0 µm wide. These cells would often become swollen with three to five PHB granules per cell. When cultured in nitrogen-deficient broth supplemented with 0.01% (wt/vol) Difco yeast extract, the cells often became long spirals of 30 to 40 µm in length. These cells had reduced motility, but were capable of rotation about their axes, and had few or no PHB granules. Peptone was found to produce similar elongated,

weakly motile cells containing little PHB. These cells were probably filaments, as described by Hughes (1956). Becking did not study their viability.

Eskew et al. (1977) isolated and studied the pigmented A. brasilense strain Cd. Nitrogenase activity peaked after about 2 days of growth in semisolid, nitrogen-free malate medium, and most cells were motile, curved rods of normal size, often containing PHB. After 3 days, however, nitrogenase activity declined sharply. By this time, the initial near-neutral pH of the medium had risen to pH 8.1. Most of the bacteria present then appeared as enlarged, ovoid, non-motile cells that were resistant to Gram-staining. The decrease in nitrogenase activity, and shift to alkaline pH, coincided with the appearance of cyst-like cells.

Tarrand et al. (1978) found that A. brasilense and A. lipoferum strains had a similar appearance after 1 day's growth in broth containing peptone, ammonium sulfate, and succinate. Most cells were short, plump, slightly-curved motile rods averaging 1.0 µm in diameter and 2.1 to 3.8 µm in length. Cell morphology changed, especially for A. lipoferum strains, when the cells were inoculated into nitrogen-free, semisolid malate medium containing 0.005% (wt/vol) yeast extract. Cells of A. lipoferum tended to increase to 1.4 to 1.7 µm in width and to 5.0 µm to over 30 µm in length. Within 1 to 2 days, many A. lipoferum cells became S-shaped or helical and retained little if any

[illegible] These [illegible] cells eventually fragmented into [illegible] ovoid cells. Many of these fragments later became large, pleomorphic cells filled with light-refractile granules, probably PHB. In contrast, A. brasilense strains transferred to nitrogen-free, semisolid malate medium initially retained their normal appearance. Only after several weeks' time in this medium did they develop some S-shaped cells and some large, pleomorphic, granule-filled forms. Falk et al. (1985) found that A. amazonense strains failed to become pleomorphic under comparable conditions.

Krieg and Dobereiner (1984) maintained that alkalinization of the medium due to oxidation of malate was responsible for pleomorphism in A. lipoferum. Cultures of this species grown in semisolid, nitrogen-free glucose medium did not become alkaline, and the cells did not become pleomorphic.

Wong et al. (1980) isolated a putative Azospirillum sp. from cellulolytic, dinitrogen-fixing mixed cultures. In semisolid, nitrogen-poor malate medium containing adequate levels of biotin, the cells were of normal size and morphology after 1 day's growth. Between the third and seventh days, the cells gradually became S-shaped and enlarged. These enlarged cells contained granules of PHB and/or polyphosphate. By 10 days, many cells had lysed and released these granules into the medium. When the initial biotin concentration of the medium was reduced to 10% of the

normal level, these morphological changes were accelerated, occurring within 2 to 3 days after inoculation. This strain could not fix dinitrogen with glucose as the carbon source, but otherwise its biotin requirement and pleomorphism were typical of A. lipoferum.

Lamm and Neyra (1981) found that A. lipoferum strains grown in nitrogen-free, semisolid malate medium developed many elongated cells after 2 days of culture, whereas A. brasilense strains only developed elongated cells after 10 days. In both semisolid and agar-plate, nitrogen-free malate cultures, thick-walled, optically refractile cells were present as 1 0% of the cells in day-old cultures of both species. After 4 days, the numbers of these thick-walled cells were equal to or greater than cells of normal morphology. The ovoid cells of A. lipoferum strains were about twice as large as those produced by A. brasilense strains. Such cells were never observed in nutrient broth cultures, but could be obtained in old cell lawns grown on nutrient agar. The increased desiccation resistance of these ovoid cells has already been discussed.

Papen and Werner (1982) observed apparent cysts of A. brasilense Sp 7. Such nonmotile cells were encapsulated, having a diameter of about 1.2 µm, were not fixing dinitrogen, and were desiccation resistant. They composed the majority of cells in the nitrogen-free, malate broth culture between the second and fourth days of incubation. After

this time, ve[illegible] emerged fro[illegible] t[illegible] grow and fix [illegible]trogen. The authors suggested that oxygen limitation greatly affected these events. The level of PHB increased as the oxygen level of the cultnre decreased; nitrogenase activity ceased and the cells encysted for a time. Their apparent reduced respiratory activity allowed the level of dissolved oxygen to be replenished in the medium, until vibrioids emerged from the cyst coats to grow and fix dinitrogen again. No encystment was observed when cultures were incubated aerobically.

The recent work of Sadasivan and Neyra (1985) stressed the roles that PHB and exopolysaccharides play in cyst formation of azospirilla. Encysting cells lost their motility and became enlarged and rounded. They accumulated PHB and synthesized capsular material. The investigators emphasized that common media, such as nutrient broth, do not promote encystment and that development of mature exine and intine layers may only be achieved under specific, well-defined cultural conditions. Sadasivan (1985) may have found the cultural conditions to promote maturation of cysts of A. brasilense Sp 7. Using phase contrast microscopy, she has observed vegetative cells emerging from cyst coats, leaving behind empty "horseshoe"-shaped capsules. She has also observed cysts containing from two to four central bodies within a single exine. In transmission electron microscopy thin sections, she has observed maturing cysts,

with membranous blebs migrating outward into the capsular material from central bodies containing PHB granules. She has also observed mature cysts with central bodies containing PHB and polyphosphate granules, surrounded by distinct intine and exine layers. Thus, given appropriate cultural conditions, A. brasilense Sp 7 is able to form apparently mature cysts, almost identical in appearance to those of Azotobacter spp. One unusual feature she has reported is layers of spherical, melanin-like granules outside the exines of mature A. brasilense cysts, these layers have never been observed with Azotobacter spp. cysts.

Berg et al (1980) studied morphological and physiological changes of A. brasilense Sp 7 grown under different conditions. Encapsulated cells (C-forms) were often present on cells lawns grown on nitrogen-free succinate agar. Encapsulation was initially heaviest for cells near the lawn surface. After most cells in the surface layers were converted to nonmotile C-forms, the lower cell layers began to accumulate capsules. Such C-forms were not observed within 60 hours of growth in semisolid, nitrogen free succinate agar. They formed rapidly on nitrogen-free agar surfaces. Most of the culture formed capsules. The appearance of the encapsulated forms varied and changed with time. Both capsule formation and PHB accumulation were inhibited by combined nitrogen. As cultures aged, enlarged vibrioid C-forms developed. The more mature forms were spheres of

2.0 to 4.0 μm diameter which had lost [illegible]. One-week-old cultures consisted mainly of spherical [illegible] of 5.0 to 8.0 μm diameter, containing many PHB-rich cells within a common capsule. The authors speculated that younger encapsulated forms may be fixing dinitrogen and that older encapsulated forms may not. They suggested that the capsule may reduce oxygen flow into the cells, thereby protecting oxygen-sensitive nitrogenase activity. Azospirilla form extensive capsules only in media having a high C/N ratio (Sadasivan and Neyra, 1985). Such conditions promote nitrogenase activity. Since most capsules contain over 95% of their weight as water (Costerton et al., 1981), and oxygen diffuses through water at one ten-thousandth the rate of diffusion through air (Clark, 1967), the capsule may well help protect nitrogenase from oxygen damage. Oxidation of PHB reserves within the cell may also reduce oxygen levels near the nitrogenase (Dawes and Senior, 1973).

The description by Berg et al. (1980) of encapsulation starting at the uppermost layers of nitrogen-free agar-grown colonies and proceeding downwards is reasonable, if one assumes that encapsulated cells are metabolically active for a time, and then pass into dormancy. Initially, the uppermost encapsulated cells would be actively fixing dinitrogen. They might become dormant as a result of underlying cell layers depleting the available carbon supply, or possibly because conditions become favorable for their passage

[illegible]titutive dormancy (Parshall 1980) [illegible]ls might [illegible]o encyst when they accumulate threshold l[illegible]els of [illegible]ular material and/or PHB. In any case, as dormant cells they would consume little oxygen, allowing it to diffuse to lower cell layers that previously may have been oxygen-limited, due to the actively respiring upper cell layer. These lower cell layers would become more active with the increased oxygen supply, accumulating capsules. Eventually these cell layers would also pass into dormancy.

In earlier work, Berg et al. (1979) grew A. brasilense Sp 7 in association with sugarcane callus tissue. Vegetative cells (V-forms) grew as slimy colonies on the surface of the callus, and few of these V-forms contained PHB or capsules. Encapsulated or C-forms were also observed in these conditions. This association of azospirilla with sugarcane callus exhibited nitrogenase activity, but whether the V-forms, C-forms, or both were responsible could not be ascertained, since both were present. Perhaps C-forms were able to fix dinitrogen transiently, but were poised to enter dormancy if growth became too unbalanced. The bacteria did not possess capsules near or within lysed plant cells, where the C/N ratio may have been narrow, and balanced growth may have been promoted.

An apparent contradiction in this plant callus-bacterium work is the claim by Berg et al. (1979) that C-forms of azospirilla have little similarity to Azotobacter

[illegible] cysts. Krieg and Dobereiner (1984) restated this, but the photographs of Berg et al. (1979) do not support it. The multicellular C-forms are virtually indistinguishable from Azotobacter spp. cysts having multiple central bodies (Cagle and Vela, 1974). Clearly, in the association with sugarcane callus, the azospirilla were situated in numerous sites, differing in nutrient availability and oxygen availability. It is not surprising that multiple morphologies were observed, reflecting multiple physiological states. Only a few cells resembling mature cysts were present.

Pleomorphic forms of azospirilla have been observed in a variety of axenic associations with plant roots. The work of Umali-Garcia et al. (1980) has already been discussed. Rusco et al. (1978) grew maize plants in sand and inoculated them with different strains of azospirilla. Enlarged, cyst-like cells, as well as cells of normal morphology, were observed in older and thicker root segments, where root tissue was often disintegrating. They also found that when two strains of azospirilla were grown in nitrogen-free, semisolid trans-aconitate agar, they often formed long chains after 4 to 5 days.

Matthews et al. (1983) used immunological techniques and transmission electron microscopy to observe strains of A. brasilense in axenic association with pearl millet roots. Both vibrioid and encapsulated cells were observed in association with the roots. The encapsulated cells

usually [illegible] PHB and polyphosphate granules, or [illegible] two or more cells were enclosed by a common capsule.

Patriquin et al. (1983) observed unusual structures on the surface of wheat roots, 3 weeks of age and older, that had been axenically incubated with azospirilla in a sand-vermiculite mix. They appeared as spherical "bags," within which azospirilla containing PHB granules could be seen to swim about. These structures were also found between the epidermis and outer cortex of young wheat roots.

Krieg and Doberelner (1984) suggest that the capsule of azospirilla helps protect nitrogenase. They also support the idea that development of alkaline pH is the cause for pleomorphism in A. lipoferum and A. brasilense. This seems an incomplete explanation, implying that pleomorphic cells are poorly viable, being aberrant forms or laboratory artifacts. Pleomorphic cells of azospirilla may instead develop commonly, and perhaps transiently, when growing in natural environments of high C/N ratio, such as near plant roots. Unbalanced growth, with increased PHB and capsule formation, may be the major cause of pleomorphism.

Experimental Approach

The conversion of 90% or more of an Azotobacter sp. cell suspension to cysts facilitates physiological studies of cysts. Growing the cells in nitrogen-free media containing n-butanol or BHB leads to this conversion.

Azospirilla [illegible] like morphology have been observed [illegible] various cultural conditions, but reports of conversion of 90% or more of cell populations to cyst-like forms are not found in the literature. Vegetative cells are reported as being present in high numbers, along with the cyst-like forms. This has perhaps discouraged studies on the nature of cyst-like forms of azospirilla.

All strains of A. brasilense and A. lipoferum are able to grow on BHB as sole carbon source in the presence of combined nitrogen (Tarrand et al., 1978). However, no studies have been done to see how azospirilla respond to BHB in the absence of combined nitrogen. Since such cultural conditions lead to prolific encystment of some Azotobacter strains, it was considered worthwhile to determine if strains of azospirilla might also undergo conversion in high numbers to cyst-like forms under these growth conditions.

The research reported here addresses the following questions:

1. Can high numbers of cyst-like forms of azospirilla be obtained by growth in nitrogen-free BHB broth or on nitrogen-free BHB agar?
2. What are the morphological differences between azospirilla grown on BHB with or without combined nitrogen?

1. Is the PHB content of azospirilla grown on NMB without combined nitrogen higher than when they are grown in complex broth with combined nitrogen?
2. If pleomorphism of azospirille occure in nitrogen free NMB broth, ie alkalinization of the medium a prerequisite for development of pleomorphism?
3. Are azospirille grown on nitrogen-free NMB ager more desiccation resistent then cells grown in complex broth with combined nitrogen?
4. Are azospirille grown on nitrogen-free NMB ager more resistent to starvation in carbon- and nitrogen-free, phosphate-buffered salts solution than cells grown in complex broth with combined nitrogen?
5. What growth conditions favor motile azospirille arising from nonmotile azospirille grown on nitrogen-free NMB ager?
6. Is protein synthesis required before nonmotile NMB-grown azospirille give rise to motile azospirilla?
7. Are NMB-grown azospirilla effected by Tris-EDTA in a manner similar to Azotobacter cysts?

Questions 1 through 4 are considered in Chapter II, and the remaining questions are considered in Chapter III.

CHAPTER II
[illegible]ORPHISM OF AZOSPIRILLA [illegible] BETA-HYDROXYBUTYRATE

Only a [illegible] bacterial genera contain strai[illegible] form cysts (Sudo and Dworkin, 1973; Whittenbury [illegible], 1970a, Tudor and Conti, 1977). A nonmotile cyst forms when the entirety of a vegetative cell rounds up, deposits extracellular coats and often accumulates intracellular energy-reserve polymers.

The morphological changes of encystment are accompanied by a reduction in cell metabolic activities, and increased resistance to environmental stresses, such as starvation and desiccation. Cysts of Azotobacter spp. are perhaps the best understood. Like other prokaryotic resting cells, they form when vegetative cells undergo a metabolic shift-down (Sadoff, 1975).

Cysts of Azotobacter spp. do not form in media supporting good vegetative growth until stationary phase, and are present then only in low numbers (Sadoff et al., 1971). Similarly, cells of azospirilla are uniform in shape during active growth in nutritionally complete media (Umali-Garcia et al., 1980; Lamm and Neyra, 1981; Sadasivan and Neyra, 1985). As is true for Azotobacter spp., however, stationary phase cultures of azospirilla grown on complete media often

contain some rounded, nonmotile cells (Lamm and Neyra 1981; Papen and Werner, 1982; Krieg and Dobereiner, 1984)

Azospirilla are morphologically vexing, in that different pleomorphic cell types occur under various growth-limiting conditions. Weakly motile filaments containing little PHB form in aerobic broth which is low in combined nitrogen (Hecking, 1982). Under dinitrogen-fixing conditions, filamentous or S-shaped cells again may arise but contain large deposits of PHB (Tarrand et al., 1978; Wong et al., 1980, Lamm and Neyra, 1981). These elongated cells often fragment into smaller, oval cells which subsequently can assume a cyst-like morphology (Tarrand et al., 1978).

The most frequently reported pleomorphic form of azospirilla is a nonmotile cell possessing thick outer layers, probably of capsular material. These cells usually contain more extensive deposits of PHB than do vegetative cells grown with combined nitrogen. These cells have been observed in older cultures grown on combined nitrogen (Lamm and Neyra, 1981), in cultures grown as dinitrogen-fixers (Eskew et al., 1977; Berg et al., 1979; Berg et al., 1980; Papen and Werner, 1982), and in xenic associations with grass roots (Rhoades et al., 1978; Umali-Garcia et al., 1980; Matthews et al., 1983). Recently, Sadasivan and Neyra (1985) obtained them in broth containing fructose and KNO_3

The nomenclature for describing these cells is not standardized. Berg et al. (1975) termed them capsulated or

forms as opposed to the vegetative or V-forms, as did some later workers (Matthews et al., 1983 Krieg and Dobereiner, 1984). This terminology may be confusing, however, since capsules can also occur on azospirilla of otherwise normal morphology (Nur et al., 1980)

The presence of a capsule is usually deemed a prerequisite for cyst formation in Azotobacter spp. (Eklund et al., 1966). Azospirilla also may need to form a capsule before they can form cyst-like cells. Encapsulated azospirilla may initially be fully active vegetative cells. Upon encountering metabolic or environmental stress, such cells may mature into cyst-like cells. The change in morphology with time of some members within a C-form population (Berg et al., 1980) may reflect maturation into truly mature cysts. Two definitive traits of a mature Azospirillum spp. cyst would be greatly reduced cell metabolism and enhanced desiccation resistance. Morphologically differentiated cells of azospirilla have been called cysts when they exhibit no nitrogenase activity (Eskew et al., 1977, Papen and Werner, 1982) or exhibit enhanced desiccation resistance (Lamm and Neyra, 1981; Papen and Werner, 1982; Sadasivan and Neyra, 1985).

Another complicating factor in understanding these forms of azospirilla is that their appearance in dinitrogen-fixing cultures often coincides with alkalinization of the growth medium (Eskew et al., 1977; Krieg and Dobereiner,

1981). Krieg and Doberelner (1984) suggest that these cell forms arise mainly at excessively high pH. In this case they might be only laboratory artifacts, or involution forms, that have no in situ function. The findings of Lamm and Neyra (1981), Papen and Werner (1982), and Sadasivan and Neyra (1985) argue against this viewpoint. Indeed, the ability of azospirilla to enter dormancy as cysts may help explain some of the great variability of plant responses to inoculation with these bacteria (reviewed by Patriquin et al., 1983).

Two things are presently lacking in research and understanding of cyst-like forms of azospirilla. Although cyst like forms of azospirilla have been predominant in some studies, growing cells of normal morphology (vegetative cells) have always been present in high numbers as well. Conversion of 90% or greater of a population of vegetative azospirilla to cyst-like forms (quantitative encystment) in a reproducible manner would greatly facilitate further study of these cell forms, as it did for Azotobacter app. cysts (Socolofsky and Wyss, 1962). Also lacking is an understanding of the underlying causes of pleomorphism and cyst formation in azospirilla.

Conversion of 90% or greater of a cell population of Azotobacter app. to cysts often can be achieved by culturing vegetative cells in the absence of combined nitrogen on either of two precursors of PHB, n-butanol or BHB (Sadoff,

1975). Although all strains of A. brasilense and A. lipoferum are known to grow on PHB as sole carbon source when provided with combined nitrogen (Tarrand et al., 1978), there are no reports of the response of azospirilla to PHB in the absence of combined nitrogen. It was thought worthwhile to see if vegetative azospirilla would respond similarly to Azotobacter spp , by undergoing quantitative encystment in the presence of these carbon sources. In preliminary studies, apparent extensive PHB accumulation and capsule formation were observed in some strains of azospirilla grown with n-butanol. Since n-butanol is volatile, PHB was used for later studies.

Initial objectives of this study were to achieve morphological encystment of high numbers of azospirilla, to document the morphology of such cells, to verify that they contained PHB, and to ascertain if alkalinization of the medium was a prerequisite for their formation.

Materials and Methods

Bacterial Strains

The Azospirillum brasilense strains used in these studies were A. brasilense strain JM 125A2 and A. brasilense strain Cd (ATCC 29729) (both courtesy of J. Milam, Univ. of Florida, Gainesville). The Azospirillum lipoferum strains used were A. lipoferum Sp RG6xx (ATCC 29731), A. lipoferum Sp RG20a (ATCC 29708), A lipoferum Sp RG8C, and

A. lipoferum Sp A3a (all courtesy of N. R. Krieg, Va. Polytech. Inst., Blacksburg). All strains were maintained on slants of Tryptic Soy Agar (Difco Laboratories, Detroit, MI) at 4-5°C with monthly transfer.

Media

Vegetative azospirilla were cultured in a modification of the complete medium of Tyler et al. (1979), denoted as trypticase-succinate salts (TSS). All components were of reagent grade and were dissolved in deionized water. The final concentrations of TSS components were (in grams per liter): $(NH_4)_2SO_4$, 0.5; succinic acid, 0.437; Trypticase Peptone (Baltimore Biological Laboratory, Cockeysville, MD), 1.0; d-biotin (Sigma Chemical Co., St. Louis, MO), 0.0001; NaCl, 0.1; $FeCl_3 \cdot 6H_2O$, 0.0017; $Na_2MoO_4 \cdot 2H_2O$, 0.0002; $MgSO_4 \cdot 7H_2O$, 0.2, and $CaCl_2$, 0.002. The first four components were omitted to obtain a basal salts solution. The biotin was dissolved as a 100X concentrated stock solution by heating and then filter-sterilized by passage through a 0.2 µm pore diameter Nalgene filter unit (Nalge Company, Rochester, NY). Two phosphate buffer concentrations were employed. The low phosphate (LP) buffer of Tyler et al. (1979) had a final concentration of 3.5 mM and consisted of (in grams per liter) K_2HPO_4, 0.1 and KH_2PO_4, 0.4. The high phosphate (HP) buffer of Albrecht and Okon (1980) had a final concentration of 63.8 mM and consisted of (in grams

[illegible] KH_2PO_4, 4.0- [illegible] LP buffer was prepared as [illegible] concentrated stock solution, and the HP buffer as a 10X concentrated stock solution. The pH of the LP buffer was adjusted to 7.1, and that of the HP buffer to 6.7, with 10 M KOH. The buffer stock solutions were sterilized by autoclaving. All autoclavings in these studies were for 15 min at standard temperature and pressure unless otherwise stated.

The TSS components, excluding the biotin and phosphates, were dissolved and adjusted to pH 7.0 with 10 M KOH. The broth was then dispensed into 250 ml Erlenmeyer flasks, in an amount calculated to obtain a final volume of 100 ml after aseptic addition of the biotin and phosphate buffer stocks to the autoclaved TSS. The initial pH of the LP-TSS was 6.8 to 7.0, and that of HP-TSS was 6.8.

Plate counts of azospirilla were performed with a modified succinate-nitrogen free (SNF) agar medium derived from Tyler et al. (1978). It was the same as LP-TSS, except that $(NH_4)_2SO_4$ and Trypticase Peptone were omitted. It contained in addition (in grams per liter) Bacto yeast extract (Difco), 0.05 and Bacto agar (Difco), 20.0. It was prepared in the same way as TSS broth, except that agar was added after neutralization and before autoclaving. Before Petri plates were poured, biotin and LP buffer were added aseptically, as was a solution of autoclaved Congo Red

(Sigma), that was incorporated at a final concentration of 0.0375 grams per liter (Rodriguez Caceres, 1982).

Nitrogen-free agar plates containing n-butanol had the same composition as SNF plates, except that the agar concentration was 1.5% (wt/vol), and yeast extract and Congo Red were omitted. The n-butanol was sterilized by filtration in the same manner as the biotin and incorporated at a final concentration of 0.2% (vol/vol). Beta-hydroxybutyrate was prepared from crotonic acid (Sigma) by dissolving 23.6 g crotonic acid in 900 ml deionized water. This solution was continuously mixed with a magnetic stirrer for two to three days at 25°C. Its OD_{235} stabilized by this time, indicating conversion to BHB (H. L. Sadoff, personal communication). It was then adjusted to pH 7.0 with 10 M KOH, the final volume made up to one liter, and sterilized by autoclaving. This served as a 10X concentrated stock solution of BHB for addition to agar or broth, to give a final concentration of 0.236% (wt/vol) BHB. Agar plates containing BHB had the same composition as n-butanol plates, except that $(NH_4)_2SO_4$ or Congo Red were sometimes added at the previously described concentrations. For two-step broth replacement studies (described below), broth contained BHB, biotin, and phosphate-buffered basal salts solution. The LP and HP buffers were employed in different broth replacement studies. The initial pH, after inoculation, of LP-BHB broth was 7.2, and that of HP-BHB broth was 6.3.

Growth Conditions

Inocula of azospirilla were grown in screw-cap tubes containing 10 ml of autoclaved Bacto Nutrient Broth (Difco) for 24-48 hours at 28°C. One milliliter of inoculum was aseptically pipetted into 100 ml of HP-TSS broth and incubated for 20 to 22 hours at 30°C at 130 rpm on a rotary shaker. By this time, the cultures attained OD_{560} readings of 0.7 to 0.9, as measured with a Bausch and Lomb Spectronic 20 spectrophotometer. The pH of the cultures at harvest ranged from 7.0 to 7.2. Cultures were pelleted by centrifugation at 6,960 X g for 15 min at 20°C. Cells were washed twice by resuspension and pelleting in sterile LP-basal salts solution (pH 7.3). The cells were resuspended in sterile LP-basal salts solution to give a final OD_{560} reading of 1.0 to 1.2.

Cell lawns were obtained by spread plating 0.1 ml of washed cells onto agar media. Inoculated plates were sealed with Parafilm and incubated at 28°C.

For two-step broth replacement studies, washed cells were aseptically added as a 10% (vol/vol) inoculum to 250-ml Erlenmeyer flasks, containing a final volume of 100 ml NMB broth after cell addition. Duplicate flasks were incubated in the same manner as TSS flasks. These studies were modeled after the two-step replacement method of Lin and Sadoff (1968).

Harvest of Cell Lawns

To harvest lawns of azospirilla grown on [illegible] or BHB agar, about 7.0 ml of sterile deionized water was aseptically poured across the surface of a cell lawn, and the cells gently scraped from the agar surface with a flamed wire loop. For PHB analyses and plate counts, the suspended cells of one BHB agar plate were aseptically transferred to another plate whose cells were in turn scraped off. This was done to ensure that the cell suspension would not become too diluted.

Enumeration

Vegetative cells from TSS-broth, or cells grown on BHB agar, were diluted ten-fold in a series of dilution blanks containing LF-basal salts solution. For enumeration, 0.1 ml of cell suspension was aseptically spread on SNF-Congo Red agar plates. Four plates were spread for each dilution. Plates were incubated as described above for 5 days before counting.

Dry Weight Determination and PHB Analysis

To assay PHB content of vegetative cells of A. lipoferum Sp RG6xx, two 22-hour-old, HP-TSS cultures ($OD_{560} = 0.6$) were pooled for centrifugation and washing as described above, except that sterile deionized water was used for washing. The final cell suspension was adjusted to

an OD_{560} - 0.86 [illegible] grown on nitrogen-free-LP-BHB agar [illegible] by scraping (described above), to give a [illegible] volume of about 100 ml. These cells were centrifuged and washed in sterile deionized water (described above) and resuspended to give an OD_{560} of 0.25 to 0.28

For dry weight determinations, 10.0 ml of the final cell suspension were pipetted into previously weighed and desiccated aluminum pans. Five replicate pans were prepared for each cell type. The pans containing cells were dried to constant weight at 100°C. Pans were kept in a glass desiccator over anhydrous $CaSO_4$ (Drierite) after removal from the oven and before weighing.

For PHB determination, 10.0 ml of washed cells were added to 15 ml Corex centrifuge tubes (Corning Glass Works, Corning, NY) and pelleted by centrifugation at 7,080 X g for 20 min at 4°C. Three replicate tubes were prepared for each cell type. The supernatant was poured off, and subsequent steps were performed by the method of Law and Slepecky (1961). Digestion of cell pellets was begun with the addition of 10 ml of Clorox bleach (5% (wt/vol) hypochlorite). Cells were suspended in the bleach with Pasteur pipettes then the tubes were capped with glass marbles and incubated in a 37°C water bath. Digestion to constant OD_{650} was monitored with a Spectronic 20 spectrophotometer and was judged to be complete after 18 hours. The insoluble cell

material was pelleted by centrifugation as a [illegible] washed once in 10 ml of sterile deionized water and pelleted again. The volume for all subsequent washings and digestions was maintained at 10.0 ml, and all chemicals were of reagent grade. The OD_{235} of the samples in the final digestion of concentrated H_2SO_4 was measured in quartz cuvettes (1.0 cm light path), using a Carl Zeiss M4QIII spectrophotometer. For the standard curve, the sodium salt of DL-β-hydroxybutyric acid (Sigma) was dissolved directly in concentrated H_2SO_4. The standard curve was linear up to 8.0 μg PHB/ml. The PHB content of cell digests was related back to dry weight values, to determine what percentage of cell dry weight was present as PHB.

Scanning Electron Microscopy (SEM)

Samples of 0.4 ml from either LP-PHB agar plates or two-step, broth-replacement cultures were employed for SEM studies. Cells were removed aseptically from the two-step, broth-replacement cultures at the same time that culture pH was measured. Cells were aseptically impinged upon autoclaved 25-mm-diameter, 0.45-μm-pore-size Nuclepore® polycarbonate filters (Nuclepore Corporation, Pleasanton, CA), housed in a filter chimney attached to a vacuum source. About 10.0 ml of sterile, deionized water was added to the chimney after cell addition, to help distribute the cells evenly over the membrane surface, then a vacuum was

exceeding 33 d kPa was applied. Filter membranes were then removed and placed into Karnovsky's fixative (1965) for 1 hour. Filter membranes were subsequently rinsed twice for 10 min in cacodylate buffer and then dehydrated in a graded series of ethanol concentrations (10, 20, 30, 50, 70, 90, 95, 100, and 100%) for 10 min at each concentration. The samples were then air dried. Sections of filters were excised, placed onto aluminum stubs with double-stick tape, and gold coated with an Eiko IB-2 coater. Specimens were examined with a Hitachi S450 scanning electron microscope at 20 kilovolts. Photographs were taken with Polaroid Type 55, positive/negative, 4X5 Land film.

Light Microscopy

Cells were routinely observed by phase-contrast microscopy using a Wild M20 or a Nikon Labophot microscope. Cell dimensions were measured with an ocular micrometer. Photographs of cells viewed with the latter microscope were taken with a Microflex APX camera attachment, using Ilford FP4 black and white film. All photos were taken using phase- contrast optics, unless otherwise indicated.

Results

Quantitative Morphological Change

In the initial phase of these studies only three strains of azospirilla were used, A. *brasilense* strain

JM 125, A. brasilense strain Cd, and A. lipoferum Sp RG6xx. Slime developed at the bottom of stationary phase LF-TSS broth cultures of all three strains. Phase-contrast-microscopy examination of A. lipoferum Sp RG6xx slime often revealed numerous, nonmotile masses of cells similar to zoogiea, surrounded by nonmotile vegetativa calls (Figure 2-1a, b). These masses were notable for their symmetrical but varied shapes. They were darker than most of the surrounding vegetative calls, perhaps indicating greater viability than that of the surrounding pale vegetative cells. These zoogleal masses retained their shape and did not fragment into individual cells when disassociated from the larger masses of cells. Similar zoogleal forms were sometimes observed in the slime of A. brasilinese strain Cd but not in that of A. brasilense strain JM 125A2. These zoogleal forms of azospirilla may be referred to as microscopic flocs, or microfloca, that are kept intact by exopolysaccharide. Although microflocs were numerous, individual normal cells were also present in large numbers under these cultural conditions.

Azospirilla were cultured as cell lawns on agar containing precursors of PHB to see if high numbers of pleomorphic forms would arise. The A. brasilense strains produced some pleomorphic forms, but cells of normal shape and size predominated, even on old lawns.

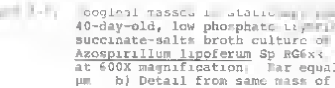
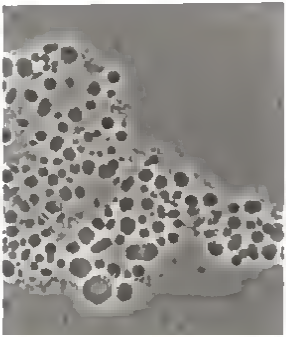
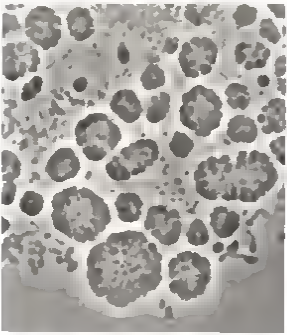


Figure 3-5. Coccoid masses in stationary phase 40-day-old, low phosphate [illegible] succinate-salts broth culture of *Azospirillum lipoferum* Sp RG6xx. a) Cells at 600X magnification. Bar equals 6.0 µm. b) Detail from same mass of cells viewed at 1,500X magnification. Bar equals 3.0 µm.



a



b

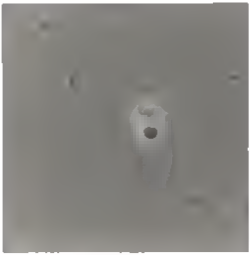
After 63 hours of growth, A. brasilense strain JM 12[illegible] lawns grown on PHB contained ovoids, vibrioids and chains of cells. Many cells contained phase-bright, putative PHB granules. More cells were present at this time on agar containing combined nitrogen. The several cell types present on 63-hour-old, LP-PHB agar with combined nitrogen are shown in Figure 2-2a. Some cells appeared at this time to be undergoing plasmolysis on this medium, as well as on HP-PHB agar with combined nitrogen. By 96 hours, the lawns on LP-PHB agar with and without combined nitrogen contained more chains of cells and microflocs than the HP-PHB lawns, which consisted mostly of individual ovoids or pairs of ovoids.

Cells from month-old, nitrogen-free, HP-PHB lawns of A. brasilense strain JM 125A2 are shown in Figure 2-2b. Individual, nonmotile vibrioids and ovoids were still predominant, as were pairs of cells. Enlarged, nonmotile, spherical cells were present, but not numerous. A few nonmotile filaments appeared to be undergoing septation.

After 79 days, cells from lawns of this strain grown with combined nitrogen had the appearance of stationary phase cells from TSS broth cultures grown with combined nitrogen, and spheroplasts and cell ghosts predominated. Nitrogen-free cultures at both phosphate buffer concentrations contained numerous pleomorphic forms. Figure 2-3a shows nonmotile, enlarged, rounded individual cells from

Cell types of *Azospirillum brasilense* strain JM 125A2, grown on β-hydroxybutyrate (BHB) agar. a) 63-hour-old cells from low phosphate-BHB agar with combined nitrogen. 1,500X magnification. Bar equals 3.0 μm. b) Month-old cells from nitrogen-free, high phosphate-BHB agar. 1,000X magnification. Bar equals 4.0 μm.

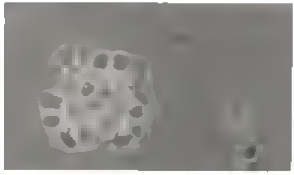
a



b

Figure 2-1. Cell types of Azospirillum brasilense strain JM 125A2, from 73-day-old nitrogen-free, high phosphate-β-hydroxybutyrate agar cultures. a) Individual rounded cells. 1,500X magnification. Bar equals 3.0 μm. b) Microfloc focused so that capsules are visible around cells on right side of floc. 1,500X magnification. Bar equals 3.0 μm. c) Same cells as (b), but focused so that capsules are no longer evident. Note empty capsule at bottom of floc. 1,500X magnification. Bar equals 3.0 μm.

a



b

c

nitrogen-free HP-BHB agar. Their phase-bright inclusions are probably PHB granules; some contain dark bodies, probably polyphosphate granules. Also shown is a microfloc of nonmotile, enlarged, PHB-rich cells (Figure 2-3b, c). By adjusting the distance of the objective lens from the specimen, many of these cells were observed to be encapsulated (Figure 2-3b). The encapsulated cells fitted together closely, as did those observed by Sadasivan and Neyra (1985). The thickness of the capsule was about 0.5 µm. Such encapsulated microflocs were also observed on nitrogen-free, LP-BHB agar at this time.

Azospirillum brasilense strain JM 125A2 may have lacked an efficient mechanism for BHB uptake, compared to the other strains of azospirilla used. Unlike the other strains, few motile cells were observed on any BHB agar medium, even in young cultures. It also differed from the other strains by having many phase-dark cells that contained little or no PHB. It eventually grew well on BHB agar when combined nitrogen was available, however. A final difference between this strain and the others was that its cells always resuspended in water to give uniform turbidity, with no macroscopic flocs, or macrofloc, being present. This indicates that, with or without combined nitrogen, cells of this strain produce little capsular material when cultured on BHB.

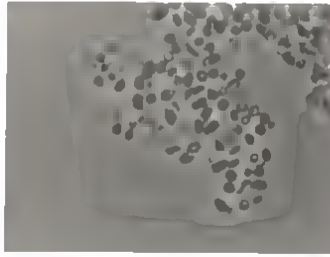
The best growth of A. brasilense strain JM [illegible] agar occurred on SNF-Congo Red agar. Cells from 5 [illegible] lawns grown on this agar medium were often seen as [illegible] sulated microflocs (Figure 2-4). The microfloc in Figure 2-4a and b appears to have arisen mainly from one or more filamentous cells that underwent septation. This may also have occurred for many of the cells in Figure 2-4c. The capsules were of thickness comparable to those observed on BHR agar, about 0.5 μm. The lawns on SNF-Congo Red agar had a scarlet or blood-red appearance, unlike lawns of this strain growing on nitrogen-free BHR-Congo Red agar, which were pale orange.

The other A. brasilense strain, A. brasilense strain Cd, also failed to convert in high numbers to pleomorphic forms, but it grew far better on BHR. After 63 hours of growth, lawns of this strain on each BHR agar medium contained many motile vibrioids possessing large granules of putative PHB. Elongated, filamentous cells were also present in high numbers. These cells had about the same width (1.5 μm) as normal dinitrogen-fixing cells but were much longer, some being 9 to 13 μm in length (Figure 2-5a). The filaments were sometimes observed to undulate slowly and were much slower than motile vibrioids. In the presence of combined nitrogen, these filaments were seen to septate and fragment. This fragmentation was observed at 63 to 96 hours, and sometimes was complete within a population of

s of Azospirillum brasilense strain J
A2, from f-day-old lawns grown on
lactate-nitrogen-free-Congo Red agar
a) Microfloc showing capsules and
filamentous cell patterns. 1,500X mag-
nification Bar equals 3.0 µm b) Same
floc as (a), but focused so that capsules
and filamentous cell outlines are no
longer evident 1,500X magnification.
Bar equals 3.0 µm c) Different mass of
encapsulated cells. 1,500X magnifica-
tion. Bar equals 3.0 µm

a ——

b ——



c ——

Cell types of Azospirillum brasilense strain Cd, from lawns on β-hydroxybutyrate (BHB) agar. a) Filaments from 63-hour-old, high phosphate-BHB agar with combined nitrogen. 1,500X magnification. Bar equals 3.0 μm. b) Microfloc from 11-day-old nitrogen free, low phosphate BHB agar, focused to show capsules and filamentous cell outline. 1,000X magnification. Bar equals 4.0 μm. c) Same floc as (b), but focused so that capsules and filamentous cell outline are no longer evident. 1,000X magnification. Bar equals 4.0 μm.

a

b

c

filaments soon after 96 hours. In nitrogen-free cultures such elongated filaments persisted, some being weakly motile even after 79 days on nitrogen-free LP-BHB agar.

After 96 hours, lawns of A. brasilense strain Cd on all BHB agar media contained mixtures of vibrioids, ovoids, filaments, and chains. Sometimes the cell material from LP-BHB agar lawns with or without combined nitrogen did not resuspend uniformly in water, but as macroflocs, due to extensive encapsulation. A microfloc from an 11-day-old, nitrogen-free, LP-BHB agar plate is shown in Figure 2-5b,c. By adjusting the objective lens, the capsule is made evident. The entire microfloc may have arisen from one elongated filament that underwent septation, as suggested by the apparent linear continuities between cytoplasmic contents.

After 79 days of culture, lawns of this strain grown with combined nitrogen contained mainly spheroplasts and cell ghosts, appearing to have entered stationary phase. Cultures grown on nitrogen-free agar at both phosphate levels contained numerous pleomorphic forms at this time, in addition to cells of normal morphology (Figure 2-6).

A microfloc of A. brasilense strain Cd from a 6-day-old lawn on SNF-Congo Red agar is shown in Figure 2-7. All the cells are encapsulated, and empty capsules are evident. The lawn was scarlet in color, unlike the pale orange lawns of the same age grown on nitrogen-free BHB-Congo Red agar.

Cell types of Azospirillum brasilense strain Cd, from 79-day-old lawns on nitrogen-free, β-hydroxybutyrate (BHB) agar. a) Multicellular packets, a chain and individual ovoids from low phosphate-BHB agar. 1,500X magnification. Bar equals 3.0 μm. b) Microfloc from high phosphate-BHB agar, focused to show capsules around several cells. Air bubble is above floc. 1,500X magnification. Bar equals 3.0 μm.

a

b

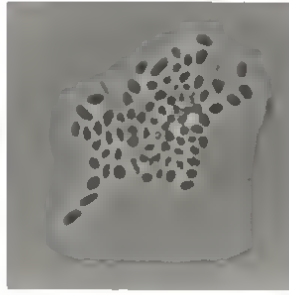


Figure 2-7. Microfloc of Azospirillum brasilanse strain Cd, from 6-day-old lawn on succinate-nitrogen-free-Congo Red agar. Note capsules around cells and empty capsules. 1,500X magnification. Bar aquals 3 0 μm

The two A. brasilense strains failed to produce [illegible] logically uniform populations on BHB-agar. A uniform response was observed for A. lipoferum Sp RG6xx. Good growth usually occurred within 18 to 24 hours. Figure 2-8 shows 18-hour-old cells grown on nitrogen-free, HP-BHB agar. Filaments and chains were present, which were some times as swiftly motile as vibrioids. On LP-BHB agar at both phosphate levels, with or without combined nitrogen, septation of filaments was almost complete between 48 and 72 hours, although new filaments would arise and septate for up to the fifth day. Figure 2-9a, b shows such completely septated microflocs on 63-hour-old, nitrogen-free, LP-BHB agar. The flocs are encapsulated, and most seemed to arise from one filament that underwent complete septation. Although microflocs were present at this time, the cells resuspended from agar as uniformly turbid suspensions without macroflocs.

Many filaments were also completely septated by 63 hours on HP-BHB agar containing combined nitrogen, but some filaments still lacked completed septa (Figure 2-9 c). Nitrogen-free, HP-BHB agar at this time contained few if any microflocs. As for all other media, very motile vibrioids and ovoids, as well as filaments of varying motility, were present at 63 hours.

Encapsulated microflocs sometimes formed on nitrogen-free, HP-BHB agar (Figure 2-10a), but cells from young or

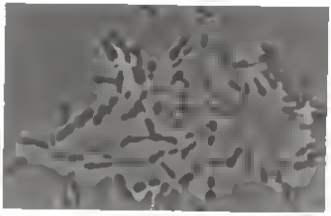


Figure 2-8. Cells of Azospirillum lipoferum Sp RG6xx, from 18-hour-old lawn on nitrogen-free, high phosphate-β-hydroxybutyrate agar. Note individual cells and filaments at various stages of septum formation. 1,000X magnification. Bar equals 4.0 μm.

Figure 2-5. Cell types of Azospirillum lipoferum Sp KG6xx, from 63-hour-old lawns on DL-hydroxybutyrate (HB) agar. a) Microflocs from nitrogen-free, low phosphate-HB agar, focused to show capsules and filamentous cell outlines. 1,500X magnification. Bar equals 3.0 µm. b) Same microflocs as (a) but focused so that capsules and filamentous cell outlines are no longer evident. 1,500X magnification. Bar equals 3.0 µm. c) Filament from high phosphate-HB agar with combined nitrogen. 1,500X magnification. Bar equals 3.0 µm

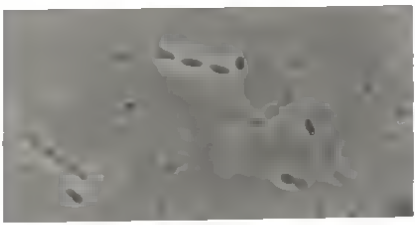
a

b

c

Figure 2-10. Cell types of Azospirillum lipoferum Sp RG5xx from lawns on β-hydroxybutyrate (BHB) agar. a) Microfloc from 13-day-old cell lawn on nitrogen free NF-BHB agar. Note empty capsules. 1,500X magnification. Bar equals 3.0 μm. b) Individual cells and septating filaments from 13-day-old, high phosphate-BHB agar with combined nitrogen. 1,500X magnification. Bar equals 3.0 μm. c) Microfloc from 79-day-old, low phosphate-BHB agar with combined nitrogen. 1,500X magnification. Bar equals 3.0 μm.

a



b

c

ata lawns grown on it were always resuspended uniformly without macrofloc. Older cultures on this medium consisted mainly of PHB-rich, nonmotile ovoids or peanut-shaped cells, with few if any microfloc. The high phosphate level did not appear to inhibit PHB accumulation, but did inhibit extensive capsule formation. As was true for the A. brasilense strains, combined nitrogen led to eventual good growth and passage into stationary phase. Figure 2-10b shows cells of a 13-day-old culture from HP-BHB agar containing combined nitrogen. Chains of cells and individual ovoids are present.

The LP-BHB lawns grown with or without combined nitrogen had the same appearance by 7 days. The lawns consisted almost entirely of flocs that broke into various sizes when resuspended in water. The cells would not suspend evenly in water, due to the presence of many macroflocs. Very few motile cells were present at this time. Eventually the LP-BHB lawns grown with combined nitrogen resumed vegetative growth and passed into stationary phase, but the flocs persisted even in stationary phase cultures (Figure 2-10c).

Figure 2-11 shows cells from 17-day-old, nitrogen-free, LP-BHB-Congo Red lawns. Figure 2-11a was taken with bright-field optics, showing the clearly outlined capsules and enlarged PHB-rich cells. Figure 2-11b was taken with phase-contrast optics, and the capsules enclosing all of the microfloc are again evident. It was interesting to find

Cell types of Azospirillum lipoferum Sp RG6xx, from 17-day-old lawns on nitrogen-free low phosphate-6 hydroxybutyrate-Congo Red agar. a) Microfloc viewed with bright-field optics. 1,500X magnification; Bar equals 3.0 µm. b) Another microfloc, viewed with phase contrast optics. Note that capsules of the two bottom-left cells are apparently undergoing division with their cells. 1,500X magnification. Bar equals 3.0 µm

a

b

that some cells that were undergoing division in one floc appeared to have their capsulas dividing as well at the site of septum formation (Figure 2-11b).

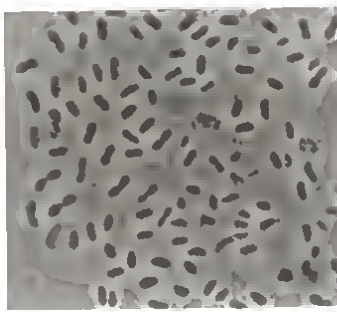
Cells from 17-day-old, SNF-Congo Red lawns are shown in Figure 2-12. Abundant capsulas are again evident. These cells appeared more vibrioid in shape than most A. brasilense cells cultured in the same manner. Cells of A. lipoferum Sp RG6xx grown on SNF-Congo Red agar appeared less swollen and rounded than their counterparts on LP-BHB Congo Red agar. However, unlike the A. brasilense strains, lawns of A. lipoferum Sp RG6xx were scarlet on both SNF-Congo Red and LP-BHB-Congo Red agar.

Nitrogen-free, LP-BHB cultures of A. lipoferum SP RG6xx did not change in appearance from the seventh day onward, even after months had passed. Figure 2-13 shows a microfloc of this strain, with the objective lens adjusted to show the capsulas (Figure 2-13a), and then readjusted to show the capsulas and the apparent continuities between cytoplasms (Figure 2-13b). Cells from this medium of 7-days-age or older were consistently resuspended as macrofloca and microflocs. Individual, septated filaments apparently consolidated into flocs, and individual, motile cells may have attached to septated filaments to give rise to large macrofloos.

More details of floc structure of A. lipoferum Sp RG6xx were obtained from SEM photographs. Figures 2-14 to

Figure 2-12. Cells of Azospirillum lipoferum Sp 6xx, from 17-day old lawns on succinate-nitrogen-free-Congo Red agar. a) Microfloc with empty capsules, as well as capsules retaining their cells. 1,500X magnification. Bar equals 3.0 µm. b) Another mass of encapsulated cells. Note that some capsules seem to contain inclusion granules but no cytoplasm. 1,500X magnification. Bar equals 3.0 µm.

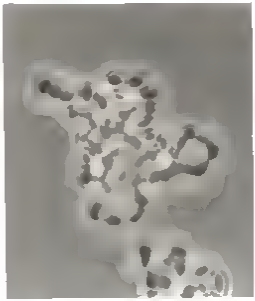
a



b

Figure [illegible] Microflocs of Azospirillum lipoferum Sp RG6xx, from 36-day-old nitrogen-free, low phosphate-β-hydroxybutyrate agar: a) Microflocs focused to show capsules. 1,000X magnification. Bar equals 4.0 µm. b) Same flocs as (a) but focused to show both capsules and filamentous cell outlines. 1,000X magnification. Bar equals 4.0 µm.

a



b

-16 show successive magnifications of cells scraped from a 75-day-old, nitrogen-free, LP-BHB agar lawn. Cells from 19-, 57-, and 66-day-old lawns on the same medium were of identical appearance to this older culture. This supported the phase-contrast microscopy studies, in that the morphology of microflocs from nitrogen-free, LP-BHB agar did not change once they were formed. The 500X magnification photo (Figure 2-14) shows the great variability in numbers of cells per floc and that individual, encapsulated cells are often present. The 1,500X-magnification photo (Figure 2-15) reveals, in agreement with the phase-contrast observations, the frequent tight fit between adjacent capsules. Intercellular gaps are often observed within flocs. The 7,000X-magnification photo (Figure 2-16) reveals some variability in the surfaces of encapsulated cells, possibly indicating a difference in exopolysaccharide composition. It is clear at this higher magnification that the cells of agar-grown flocs range from monolayers to trilayers. Empty capsules are also visible.

Similar results were obtained with this strain on LP-n-butanol agar. Figure 2-17 shows 178-day-old cells from this agar medium after the agar had dried into a thin film. When the agar surface was rehydrated and the cells scraped from it, many empty capsules were seen, but many capsules still contained cells. The capsular material was thus observed to retain its outline, even if it contained no cell

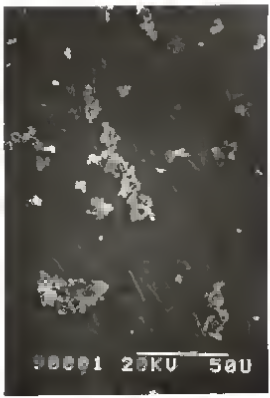


Figure 3-14 Encapsulated cells of Azospirillum lipoferum Sp RG6xx from a 75-day-old lawn on nitrogen-free, low phosphate-P-hydroxybutyrate agar. Cells are viewed at 500X magnification by scanning electron microscopy. Bar equals 50.0 µm.

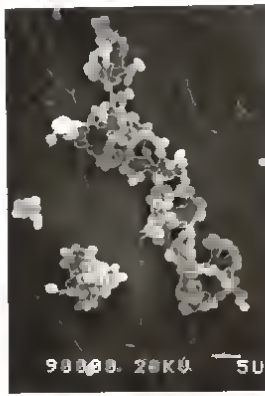


Figure 2-15. Encapsulated cells of Azospirillum lipoferum Sp RG6xx from a 75-day-old lawn on nitrogen-free, low phosphate-β-hydroxybutyrate agar. Cells are viewed at 1,500X magnification by scanning electron microscopy. Bar equals 5.0 μm.

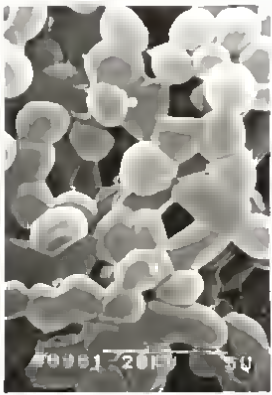
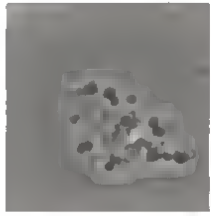


Figure 2-16. Encapsulated cells of Azospirillum lipoferum Sp RG6xx from a 75-day-old lawn on nitrogen-free, low phosphate-β-hydroxybutyrate agar. Cells are viewed at 7,000X magnification by scanning electron microscopy. Bar equals 5.0 μm.

Figure 4.7 Microfloes of Azospirillum lipoferum Sp RG6xx from 178-day-old lawns grown on nitrogen-free, low phosphate-n-butanol agar a) Microfloc showing numerous cells within capsules as well as empty capsules. 1,000X magnification Bar equals 4.0 µm. b) Microflocs with few cells remaining within capsules 1,000X magnification. Bar equals 4.0 µm.



a

b

Growth of this A. lipoferum strain on nitrogen-free LP-BHB agar resulted in homogeneous encapsulation and filament formation. But, as is evident from the photographs, several cell shapes and sizes were present within any one floc. Despite their morphological heterogeneity, cells in these flocs generally appeared to be more rounded and swollen than cells grown on SNF-Congo Red agar, although capsules were of equal width (0.5 µm) under both cultural conditions.

The homogeneous encapsulation and filament formation of A. lipoferum Sp RG6xx prompted a search for similar response in other strains of this species. Three other strains of this species, cultured on LP-BHB nitrogen-free agar, responded about as well as A. lipoferum Sp RG6xx (Figures 2-18 to 2-20). Cells of all strains did not resuspend uniformly in water, due to macroflocs. As was true for A. lipoferum Sp RG6xx, the appearance of the other three strains did not change noticeably with time, and several cell sizes and shapes were usually present within any one floc. Azospirillum lipoferum Sp A3e differed from all the other A. lipoferum strains in consistently having large numbers of individual, PHB-rich, nonmotile, nonencapsulated, ovoid cells in its resuspended lawns. Possibly many of these individual cells were initially present within capsules, but were released from capsules upon the addition of water. The other strains had only a few free,

Figure 2 1. Microfloca of Azospirillum lipoferum Sp RG8c, grown on nitrogen-free, low phosphate-β-hydroxybutyrate agar. a) Microfloc from 69-day-old lawn, focused to show capsules and filamentous cell outline 1,500X magnification. Bar equals 3.0 μm. b) Same floc as (a) but focused so that capsules and filamentous cell outline are no longer evident. 1,500X magnification Bar equals 3.0 μm. c) Microfloca from 136-day-old lawn Note empty capsules. 1,000X magnification. Bar equals 4 0 μm

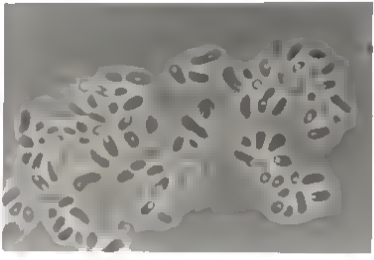
a

b

c

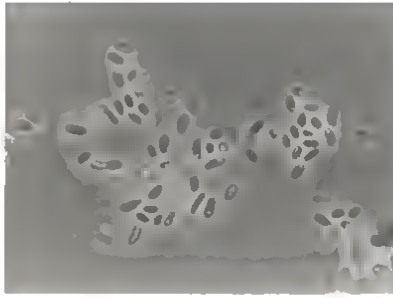
Figure [illegible]. Microflocs of Azospirillum lipoferum Sp RG20a, from 69-day-old nitrogen-free, low phosphate-β-hydroxybutyrate agar: a) Microflocs focused so capsules and filamentous cell outline are evident in upper left part of left floc. 1,500X magnification. Bar equals 3.0 μm. b) Microflocs with cells having varied morphologies. 1,500X magnification. Bar equals 3.0 μm.

a

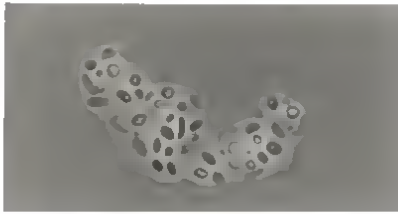


b

Microflocs of Azospirillum lipoferum Sp A3a from nitrogen-free, low phosphate-β-hydroxybutyrate agar. a) Microfloc from 69-day-old lawn. 1,500X magnification. Bar equals 3.0 μm. b) Microfloc from 136-day-old lawn. Note empty capsules. 1,000X magnification. Bar equals 4.0 μm.



a —



b —

nonencapsulated cells in their suspensions. Whether these free cells never merged with microflocs, or whether they were ejected from their capsules upon wetting and suspension for microscopy, is not clear. The width of the capsules on these three strains was again observed to average about 0.5 μm, and most flocs arose from one or more filaments that eventually underwent septation.

Enumeration of Encapsulated Cells

It was first suspected that cells of A. lipoferum Sp RG6xx harvested from nitrogen-free, LP-BHB agar would not be quantifiable by plate counting. Plate counts demand that the inoculum be uniformly suspended and diluted, and flocculation makes these difficult to accomplish. But the resuspended cells gave consistent CFU counts for a given OD_{560} range (Table 2-1). After resuspension in water and washes in buffer, the cell suspensions appeared silvery, and floc size was reduced to the lower limits of visibility to the naked eye. Macrofloes broke into smaller microfloc domains, which had formed within the first few days on the agar medium. These small domains retained their integrity even after repeated shaking and washing steps. The similar CFU counts for cells of different ages suggested that the cells were in a sort of stasis, where they were no longer multiplying or dying off appreciably.

...tical density (OD_{560}) and colony forming units ml^{-1} (CFU ml^{-1}) of encapsulated cells of Azospirillum lipoferum Sp RG6xx.

Culture age (days)[a]	OD_{560}	CFU ml^{-1} (X 10^6)
1	0.27	2.67 (0.12)[b]
1	0.28	5.08 (0.79)
2	0.25	6.40 (1.00)
58	0.24	1.94 (0.19)
75	0.25	2.11 (0.32)
80	0.30	6.65 (0.49)

[a]Cells were harvested from nitrogen-free, low phosphate-β-hydroxybutyrate agar plates.

[b]Values are averages of four spread plates. Values in parentheses are standard deviations.

Poly-β-Hydroxybutyrate Content

The phase-bright inclusion bodies in the cells of A. lipoferum Sp RG6xx grown on nitrogen-free, LF-BHB agar were confirmed to be PHB using the method of Law and Slepecky (Table 2-2). This method is subject to error due to repeated centrifugations and pipettings, so it is not certain whether the differences in PHB content between cell lawns of different ages were real or artifacts. The purpose was to verify that PHB existed in the encapsulated cells in greater amounts than in vegetative cells grown in HF-TSS broth. The assay gave evidence of this.

Two-step Broth Replacement Studies

Strains of A. lipoferum cultured in LF-TSS broth clumped and flocculated in under 24 hours. Clumping of these strains was delayed in HF-TSS broth. The pH of these cultures at 20 to 22 hours ranged from 7.0 to 7.2, having risen from an initial pH of 6.8. Cells of each A. lipoferum strain in this study, grown in HF-TSS broth, usually started to clump and flocculate by about 24 hours after inoculation. Figure 2-21 shows a SEM photo of cells from a 14-day-old, HF-TSS culture of A. lipoferum Sp RG6xx. The pH of the culture at this time was 9.1. The floc has a similar arrangement to microflocs of the same strain grown on BHB agar, with filamentous cells and frequent spaces in the

2-2 Poly-ß-hydroxybutyrate (PHB) [illegible] in Azospirillum lipoferum Sp RG6xx.

[illegible]rural [illegible]itions	Age	Dry weight (mg ml^{-1})[a]	Volume of $CHCl_3$ extract[3] used (ml)	µg ml^{-1} PHB[b]	% dry weight as PHB
[illegible]	11 days	0.17 (0.02)	0.5[d]	3.33 (0.52)	39.9
[illegible]	21 days	0.18 (0.05)	0.5[e]	4.56 (0.33)	50.7
[illegible]	2 days	0.12 (0.04)	0.5[f]	3.45 (0.03)	57.5
[illegible]	42 hours	0.49 (0.03)	3.0[e]	5.28 (0.08)	3.6

[a]Values are averages of five replicates. Values in parentheses are standard deviations.

[b]Two H_2SO_4 replicates of each $CHCl_3$ replicate were used. Values are averages of all H_2SO_4 replicates. Values in parentheses are standard deviations.

[c]1 = Nitrogen-free, low phosphate-ß-hydroxybutyrate agar.

[d]Four replicate $CHCl_3$ extracts were used.

[e]Three replicate $CHCl_3$ extracts were used.

[f]Two replicate $CHCl_3$ extracts were used.

[g]2 = High phosphate-trypticase-succinate-salts broth

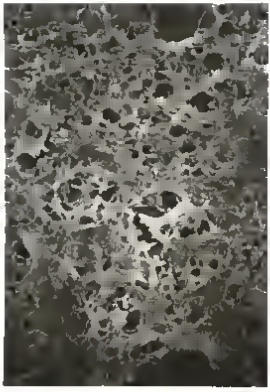


Figure 2-21. Cells of Azospirillum lipoferum Sp RG6xx, from 14-day-old, stationary phase, high phosphate-trypticase-succinate-salts broth culture of pH 9.1. Cells are viewed at 1,500X magnification by scanning electron microscopy. Bar equals 5.0 μm.

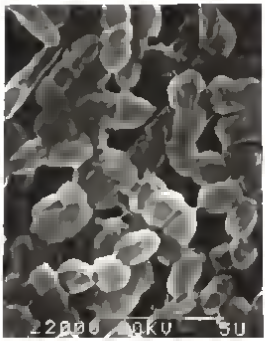
clumps. Sometimes flocs from HP-TSS broth [illegible] such empty spaces.

Two-step, broth-replacement studies were conducted to see if quantitative pleomorphism could be induced in broth and if there was any connection between pH and pleomorphism. Sometimes such cultures of A. lipoferum strains would clump extensively within 24 hours, so that the broth appeared clear to the naked eye except for the clumps, but this phenomenon was not consistently reproducible. It seemed that clumping occurred sooner and persisted longer in two-step, broth-replacement studies with A. lipoferum Sp RG6xx using LP buffer. When HP buffer was used, clear broth columns often became turbid eventually.

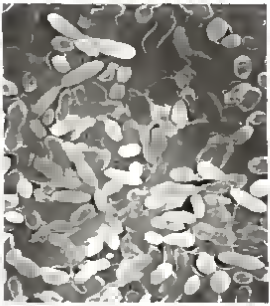
Figure 2-22a shows cells of A. lipoferum Sp RG6xx from a 43-day-old culture in LP-BHB broth of pH 8.4. The cells appear healthier than stationary phase cells in TSS broth. Filamentation and complete septation of cells occurred in most cases. The elevated pH of the culture again indicated the low buffering capacity of the LP buffer.

The results from HP-BHB, two-step, broth-replacement studies indicated that pleomorphism and encapsulation could occur at near-neutral pH for the A. lipoferum strains. All the following studies were conducted in HP-BHB broth. Figure 2-22b shows cells from a 43-day-old culture of A. lipoferum Sp RG6xx of pH 7.2. In interpreting this photo, it should be remembered that the cells were added by

Cells of Azospirillum lipoferum Sp RG6xx from 43-day-old, nitrogen-free, β-hydroxybutyrate (BHB) broth cultures, viewed by scanning electron microscopy a) Cells from low phosphate-BHB broth, pH 8.4. 7,000X magnification Bar equals 5.0 µm. b) Cells from high phosphate-BHB broth, pH 7.2 Note thorough encapsulation of lower cell layer 3,000X magnification Bar equals 5.0 µm.



a



b

pipette to the filter membrane. It appears that large, encapsulated cell flocs settled first onto the filter followed by free, nonflocculated cells. The lower layers of cells have the same close-fitting appearance as was often observed on DHB agar surfaces.

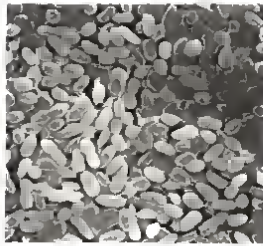
Similar results were obtained for the other three strains of A. lipoferum. Figure 2-23a shows cells from a 33-day-old culture of A. lipoferum Sp A3a of pH 7.2. Some encapsulated cells in the lower cell layer are fitted together snugly, while others are joined by strands of putative exopolysaccharide. Figure 2-23b is a higher magnification of cells from this culture, again showing the strands joining cells. The lumpy appearance of the cells is probably due to large, intracellular accumulations of PHB.

Figure 2-24 shows cells from a 33-day-old culture of A. lipoferum Sp RG20a of pH 7.2. The formation of filaments and eventually chains was very pronounced in this strain.

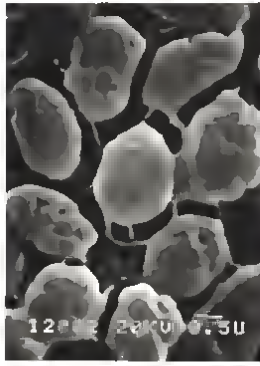
Cells from a 33-day-old culture of A. lipoferum Sp RG3a of pH 7.1 are shown in Figure 2-25a. This strain was sometimes observed to form intricately structured clumps. Figure 2-25b shows such a clump from a 9-day-old culture of pH 7.0. Filamentous, septate cells are present, and again empty spaces occur within the floc.

The two A. brasilense strains used in this study responded poorly to two-step broth replacement. The pH of

Cells of Azospirillum lipoferum Sp [illegible] from a 33-day-old, nitrogen-free, [illegible] phosphate-β-hydroxybutyrate broth culture, pH 7.2, viewed by scanning electron microscopy. a) Lower cell layer is thoroughly encapsulated. 3,000X magnification. Bar equals 5.0 μm. b) Details of cell surfaces. Note strands of material joining some cells. 17,000X magnification. Bar equals 0.5 μm.



a



b

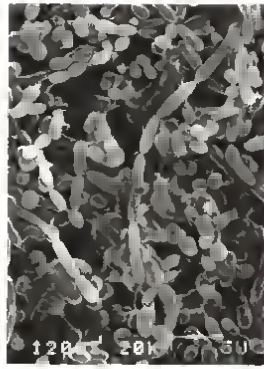
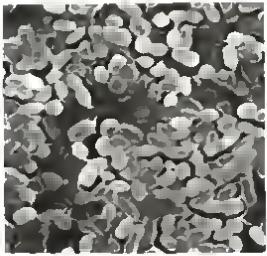
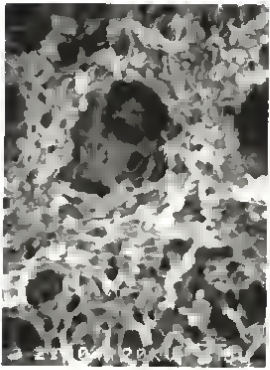


Figure 2a-d. Cells of Azospirillum lipoferum Sp RG20a from a 33-day-old, nitrogen-free, high phosphate-β-hydroxybutyrate broth culture, pH 7.2, viewed by scanning electron microscopy. Note thorough encapsulation of lower cell layer and presence of filaments and chains. 3,000X magnification. Bar equals 5.0 µm.

Figure 2-25. Cells of Azospirillum lipoferum Sp RG from nitrogen-free, high phosphate-β-hydroxybutyrate broth, viewed by scanning electron microscopy. a) Cells from 33-day-old culture, pH 7.1. Note thorough encapsulation of lower cell layer. 3,000X magnification. Bar equals 5.0 μm. b) Cells from 9-day-old culture, pH 7.0. Note holes within the floc. 1,700X magnification. Bar equals 5.0 μm



a



b

these cultures at 9 days was 6 [illegible] When viewed by SEM, cells of A. brasilense strain Cd were usually rods which had collapsed or shrunken during fixation and dehydration. Cells of A. brasilense strain JM 125A2 usually appeared as very small ovoids, having the appearance of starved or stationary phase cells. A small number of cells of the latter strain were enlarged ovoids and possibly were encapsulated. Unlike all strains of A. lipoferum tested, the two A. brasilense strains showed little or no tendency to clump in BP-PHB, two-step, broth-replacement studies.

One more observation made during BP-PHB, broth-replacement studies deserves mention. It was suggested earlier that some filamentous cells that had undergone septation and encapsulation might retain cytoplasmic connections between adjacent cells. Figure 2-26 shows a clump of cells from a 58-day-old, BP-PHB broth culture of A. lipoferum Sp RG6xx. Several of the cells in the clump appear to be undergoing plasmolysis, but there appear to be continuities of cytoplasm between some neighboring, plasmolyzed cells.

Discussion

Only two A. brasilense strains were employed in this study, and both responded far less uniformly to growth on BHB than did the A. lipoferum strains. There may have been poor uptake of BHB by A. brasilense strain JM 125A2. Cells of this strain that were able to grow on BHB

Figure 2-26. Encapsulated cells of Azospirillum lipoferum Sp AG6xx from a 50-day-old nitrogen-free, high phosphate-β-hydroxybutyrate broth culture. Note apparent continuities between cytoplasms of several cells that appear to have undergone plasmolysis. 1,500X magnification. Bar equals 3.0 μm

accumulated PHB and capsular material and sometimes [illegible] rounded and nonmotile. Better uptake and growth on BHB occurred with A. brasilense strain Cd. This strain was notable for its formation of numerous weakly motile filaments that sometimes underwent septation and fragmentation into individual cells. The presence of combined nitrogen still allowed PHB accumulation and filament formation of A. brasilense strain Cd, as was also true for A. lipoferum Sp RG6xx. Both A. brasilense strains showed some tendency to form encapsulated microflocs arising from one or more filaments that had undergone septation.

The A. brasilense strains and A. lipoferum Sp RG6xx both developed scarlet coloration when cultured as lawns on SNF-Congo Red agar. Sadasivan and Neyra (1985) have shown that azospirilla can produce cellulose as one component of their exopolysaccharides. Congo Red is known to stain many polysaccharides, including cellulose (Nakamichi et al., 1974), and colonies of azospirilla grown as dinitrogen-fixers on agar surfaces take up the dye more avidly than other free-living, dinitrogen-fixing prokaryotes (Rodriguez Caceres, 1982). It is suggested that Congo Red may profitably be used more often in cultural studies with azospirilla to examine conditions promoting capsule formation. This study indicated that nitrogen-free lawns of A. lipoferum Sp RG6xx produced capsules extensively when cultured on succinate or BHB, whereas both A. brasilense

strains [illegible] to produce capsul[illegible]y o
succinate.

All the strains of A. lipoferum use[illegible]y responded uniformly when cultured as lawn[illegible]trogen-f[illegible], LP-BHB agar. They appeared to accumulate PHB and grow as filaments that gradually lost motility [illegible]hin 5-7 days, these filaments had accumulated capsular material and become septated. By this time the cells sometimes stuck tenaciously to the agar surface and to glass surfaces as well, and macrofloc were produced. The response could be said to be homogeneous in that over 90% of the cells present were encapsulsted microflocs, but the cells themselves were not morphologically homogeneous. All contained large accumulations of PHB, but the size and shape of cells varied.

The most extensive studies were done with A. lipoferum Sp RG6xx, but its behavior was typical of other A. lipoferum strains. The appearance of the microflocs did not change noticeably with time when viewed by SEM or phase-contrast microscopy. The PHB content of the cells also remained about the same over time. It would be interesting to see if these microflocs have lower respiration than comparably cultured cells of the same age on SNF agar. The fact that lawns from nitrogen free, LP-BHB agar consistently gave CFU counts of the same order of magnitude at different culture ages for equivalent OD_{560} readings indicates that

[illegible] actively divi[illegible]ter perhaps a week's time

Pat[illegible] 1983) observed spherical, bag-like structur[illegible] and interiors of 3-week-old and older wheat roots in axenic association with azospirilla. Azospirilla containing PHB granules moved actively within these structures. These structures were similar to the zoogleal-type microflocs observed in LP-TSS broth cultures that had passed into stationary phase. It is probable that these zoogleal forms arose mainly through filamentation, followed by septation. Also reported in the previous study were sharply defined, small colonies of azospirilla of apparently determinate size on the surfaces of wheat roots. This is a good description of the microflocs of A lipoferum from nitrogen-free, LP-BAS lawns. The formation of filaments (Tarrand et al., 1978) and chains (Ruscoe et al., 1978) of azospirilla have also been previously reported.

Most laboratory studies of bacteria use cultures in a state of balanced growth, where every component of the cell culture increases at the same rate. This is done for reproducibility of results and standarization of conditions. Cultures that have passed into stationary phase have experienced a metabolic shift-down, growing more slowly and with more widely variable characteristics than log phase cells (Ingraham et al., 1983). It would appear that

Azospirilla do not become pleomorphic during [illegible] grown with combined nitrogen. Pleomorphic forms are [illegible]y observed in such cultures in stationary phase (Lamm and Neyra, 1981, Papen and Werner, 1982, Krieg and Dobereiner, 1984), after a nutritional down-shift has occurred. Media with high C/N ratios have more often resulted in pleomorphism of azospirilla (Tarrand et al., 1978; Papen and Werner, 1982 Sadasivan and Neyra, 1985). Such conditions generally promote the formation of PHB (Dawes and Senior, 1973) and exopolysaccharides (Sutherland, 1977; Costerton et al., 1981) by bacteria. High C/N ratios may also foster unbalanced growth of azospirilla, leading to pleomorphism. The formation of PHB and capsules by azospirilla seems to lead to a pleomorphic cell type that may reach maturity as a cyst.

In these experiments, azospirilla inocula were grown in HF-TSS broth containing combined nitrogen. It is probable that, in this rich medium, the cells had most of their biosynthetic operons repressed, so their biosynthetic enzymes were present only at low levels. After harvest and washing, these cells were exposed to a new carbon source (BMB) and deprived of combined nitrogen, forcing the cells into a severe metabolic shift-down requiring synthesis of biosynthetic enzymes (Ingraham et al., 1983). The azospirilla in two-step, replacement cultures were starved for nitrogen, since azospirilla cannot fix dinitrogen

aerobically. The A. lipoferum strains remained quite healthy in appearance in SEM studies under these conditions. Their ready uptake of PHB leading to PHB accumulation may have allowed them to retain their cellular integrity under conditions of nitrogen starvation. The A. brasilense strains in two-step, broth-replacement cultures did not produce visible exopolysaccharides in SEM studies. The collapsed and shrunken appearance of the A. brasilense strains in SEM studies also suggests that they were not extensively accumulating PHB.

Following completion of these studies, it was learned that the high level of phosphates used in HP-BHB, two-step, broth-replacement studies can inhibit growth of azospirilla in the absence of combined nitrogen (Scott et al., 1979; Das and Mishra, 1984). However, the A. lipoferum strains used in these studies still accumulated PHB and capsules in the two-step, broth-replacement studies where this buffer was used. These studies also showed that encapsulation and pleomorphism can occur at near-neutral pH for some A. lipoferum strains. The accumulation of PHB occurred on both HP-BHB and LP-BHB agar. Extensive encapsulation only occurred on nitrogen-free, LP-BHB agar, where the pH may have become alkaline. Alkaline pH may prevent pleomorphic cells from resuming vegetative growth.

The sequence of events for A. lipoferum strains grown in BHB broth or on BHB agar was PHB accumulation, followed

by filamentation and septation. Cells gradually lost their motility during this time. The septating filaments later produced extensive capsular material on nitrogen-free, LP-BHB agar, and sometimes in HP-BHB broth. The HP buffer repressed extensive capsule formation on nitrogen-free, HP-BHB agar.

These and previous studies indicate that pleomorphic cells of azospirilla arise under two different conditions. After cells have experienced balanced growth with combined nitrogen, some may become pleomorphic when a nutrient essential to growth becomes limiting. The production of both filamentous cells and exopolysaccharides seemed to occur extensively in HP-TSS broth in stationary phase, but PHB accumulation was not extensive.

Azospirilla may also become pleomorphic during growth where the C/N ratio of nutrients available to the cells is high. Dinitrogen-fixing cells may be poised to become pleomorphic. It is also evident that azospirilla may take up some carbon sources more rapidly than they can utilize combined nitrogen, resulting in extensive PHB accumulation and capsule formation even when combined nitrogen is available. Cells under these conditions may experience a temporary shift-down, until enough combined nitrogen can be assimilated to mobilize their PHB deposits and enable further growth. This would explain the morphological changes observed in this study with A. brasilense strain Cd

and A. lipoferum Sp RG5xx when they were cultured with BHB and combined nitrogen

The environment near plant roots, where azospirille are most often found, can be expected to provide available nutrients having a high C/N ratio (Griffin et al., 1975, Beck and Gilmour, 1983; Kreffczyk et al., 1984). Depletion of nitrate from the root zone by physical factors and plant uptake (Okon and Hardy, 1983), in combination with denitrification (Smith and Tiedje, 1979), can be expected to further elevate the C/N ratio of nutrients available to azospirilla. These bacteria have recently been found to grow and fix dinitrogen with straw, which again has a high C/N ratio (Halsall et al , 1985). Azospirilla associated with plant material can be expected to possess capsules and PHB, and sometimes to assume pleomorphic cell shapes.

As mentioned before, the most often-reported, pleomorphic form of azospirille has been a rounded, nonmotile cell possessing a capsule and PHB granules. Individual vegetative cells can likely assume this form without being a member of a microfloc or microcolony. Such rounded cells were sometimes observed within microflocs or after they had broken free of such flocs. But the pleomorphic cell types most often observed were filaments or chains of cells that eventually formed the microflocs. Bacterial filaments can arise when cells are growing very rapidly (Slater and Schaechter, 1974), or when the growth rate shifts down

[illegible] Studies reported here [illegible] azospirilla may form them under both conditions.

Strains of A. lipoferum grown in broth containing combined nitrogen have a greater tendency to clump than do strains of A. brasilense (Krieg and Doberreiner, 1984). In this study, the SEM photographs of stationary phase HP-TSS broth cultures of A. lipoferum Sp RG6xx indicated there is some structural regularity in clumps. The cells were often arranged so that the flocs contained spaces. The existence of spaces was more regular and pronounced in flocs of cells cultured with DNB in the absence of combined nitrogen, probably because the exopolysaccharides of these cells were more rigid than the exopolysaccharides of stationary phase cultures grown with combined nitrogen, which tended to be slimy

The formation of such microflocs may provide some advantages for azospirilla in nature Bergersen (1984) discussed the strategies that microaerophilic, dinitrogen-fixing bacteria such as azospirilla may have to protect their oxygen-sensitive nitrogenase. His suggestions are incorporated below into some of the advantages that azospirilla may find in growing as encapsulated microflocs:

1. The capsules may help to regulate the availability of oxygen to dinitrogen-fixing cells Assuming the capsules to be highly hydrated (Costerton et al., 1981) and assuming that water reduces the

diffusion of oxygen by a factor of 10,000 (Clark, 1967), such a role for capsules is not improbable. If the capsular material remains pliable, the cells within an encapsulated microfloc may be able to move closer together or further apart as the situation requires. When oxygen is in excess, they may move closer together, reducing the oxygen tension in one spot and thus allowing continued nitrogenase activity at that localized site. When oxygen is limiting, the cells may move further apart, the seperation allowing each cell's nitrogenase to remain functional.

The spaces between encapsulated cells may provide sites for other bacteria to enter into intimate association with the microfloc, to act in cross-feeding, or to help reduce local oxygen tension.

Encapsulation would provide the general benefits to azospirilla that most bacteria seem to derive from encapsulation (Costerton et al., 1981). These benefits include protection from predation and enhanced nutrient accumulation and uptake. The spatained rigid structure of microflocs suggests that nutrients within the encapsulated microflocs may be sequestered from the surrounding environment, giving the azospirilla a storehouse

of nutrients that may not be readily available to microbial competitors.

The encapsulated microfloc may become a fixed site where azospirilla are sometimes able to outcompete motile bacteria for carbon sources. For example, if such a microfloc became established on a root surface, it might be able to continually deplete the carbon supply from that area of root by assimilating it into PHB, without need for further cell division to occur immediately. Competing bacteria without the ability to accumulate PHB might be limited to growing at sites on the root where only balanced growth could occur.

If a microfloc is faced with starvation for exogenous carbon sources, some of its members may serve to feed others. If all septa between cells in a microfloc are completed, it could be that these septa are lysed during starvation, so that the substance of dead cells becomes available to healthier cells within the microfloc.

Most microflocs contained several different cell morphologies. Septation almost always resulted in cells of significantly different sizes and shapes. Thus, cells within a microfloc formed from a single filament may be destined to attain different physiological states after septation is

completed. Some may be poised to become actively motile as soon as exogenous nutrients enabling balanced growth become available. Others may be primed for continued dinitrogen-fixation, and still others might enter a truly dormant, cyst like state. Most microflocs observed contained at least one rounded cell that may have been cyst-like. Such a diversity of physiological states within a microcolony may help ensure the persistence of the colony at that site, providing a multiplicity of possible rapid cell responses to environmental conditions.

Although these studies failed to produce apparent quantitative, morphological encystment of azospirilla, quantitative encapsulation and microfloc formation of the A. lipoferum strains were obtained. Encapsulation seems to be a prerequisite for encystment in Azotobacter spp. (Eklund et al., 1966). It may be that encysted forms of azospirilla could be obtained in quantity by further nutritional manipulation of the encapsulated cells.

CHAPTER III
PHYSIOLOGICAL PROPERTIES OF ENCAPSULATED [illegible]
AZOSPIRILLUM LIPOFERUM Sp RG6x

In Chapter II, a method was described for q[illegible]tively converting vegetative, cell-lawn inocula of A. lipoferum strains into nonmotile, encapsulated cells having extensive, intracellular PHB deposits. Although many of the cells within encapsulated flocs had vibrioid or ovoid morphologies, some were rounded and cyst-like in appearance. Mature cysts have a lower endogenous metabolic rate and greater ability than do vegetative cells to survive carbon starvation (Sudo and Dworkin, 1973). Mature cysts of Azotobacter spp. are known to be more resistant than vegetative cells to environmental stresses, including desiccation (Socolofsy and Wyss, 1962). Such mature cysts germinate in phosphate buffer containing certain carbon sources, but not in carbon-free phosphate buffer containing ammonium, or in unamended phosphate buffer (Loperfido and Sadoff, 1973). The central bodies of mature cysts are violently and almost immediately expelled from their cyst coats when cysts are suspended in Tris-EDTA (Socolofsky and Wyss, 1961; Goldschmidt and Wyss, 1966; Lin and Sadoff, 1969; Page and Sadoff, 1975).

[redacted] if the cells of encapsulated [redacted] A. lipoferum Sp RG6xx were more resistant to desiccation and carbon starvation than were motile, vegetative cells. It was also of interest to define the conditions under which germination occurred, defined as motile cells arising from nonmotile, encapsulated microfloes. Microfloes were exposed to treatment that results in the rupture of capsular coats of mature Azotobacter spp. cysts. Finally, whether chloramphenicol inhibited production of motile cells from a nonmotile encapsulated inoculum was studied. All of these assays represented attempts to determine if flocs of A. lipoferum Sp RG6xx contained significant numbers of physiologically cyst-like cells.

Materials and Methods

Bacterial Strain

The only strain used in these studies was Azospirillum lipoferum Sp RG6xx. Its maintenance and subculture were as described in Chapter II.

Growth Media and Enumeration

Vegetative cells were cultured as previously described in the modified complete medium of Tyler et al. (1979), using the MP buffer of Albrecht and Okon (1980). Encapsulated lawns of the bacterium were cultured on nitrogen-free,

LP-MMM agar, as described in Chapter II. Plate counts were performed on the modified SNF agar medium using LP buffer and Congo Red, as described in Chapter II.

Harvest of Cells

Harvest and washing of vegetative broth cultures always employed sterile LP-basal salts solution (pH 7.3), as described in Chapter II. Lawns on LP-MMM agar were harvested in two ways. When cells were to be added to semisolid agar or assayed for desiccation and starvation resistance, they were harvested and washed in sterile LP-TSS salts solution. For every other assay that was performed, the cells were harvested and washed in sterile deionized water.

Desiccation Resistance

Vegetative HP-TSS cultures were grown for 17-22 hours, attaining an OD_{560} of 0.5 in each experiment. The cells were then centrifuged and washed twice in sterile LP-basal salts solution, as described in Chapter II. The cells were resuspended in a third volume of LP-basal salts solution to attain an OD_{560} of 0.3 in one experiment and 0.6 in another experiment. Then ten-fold dilution series in sterile LP-basal salts solution were prepared aseptically from the cells. A 0.1-ml portion of the 10^{-2} dilution was aseptically added to each of three autoclaved 10-ml glass beakers,

[illegible] autoclaved 80-mm X 10-mm Pyrex storage dish (Fi[illegible] Scientific Company, Fair Lawn, NJ). The beakers had been washed in 3.7% (vol/vol) HCl for 24 hours, then rinsed in several changes of deionized water before autoclaving.

Encapsulated cells grown on nitrogen-free, LP-SMB agar were harvested and washed in sterile, LP-basal salts solution as described in Chapter II. Cells of 75 days of age were resuspended to give a final OD_{560} of 0.25 in one experiment, and cells of 53 days of age were resuspended to give a final OD_{560} of 0.3 in another experiment. A 0.1-ml portion of cell suspension was aseptically added to each of three, 10-ml beakers housed in a storage dish as described above. Dilution series for plate counts were also prepared as for the vegetative cells. The storage dishes containing cells were placed in a glass desiccator over Drierite at 25°C for 24 hours, by which time the cell suspensions had dried onto the glass surfaces. The desiccator was then placed in a 30°C incubator for 8 days.

Initial cell numbers before drying treatment were enumerated by spread plating. For enumeration of cells surviving the desiccation treatment, the beakers were removed from their storage dishes in a laminar flow hood. The dried cell films were outlined with an ink marker to help ensure that they would be resuspended. A volume of 2.0 ml of sterile, LP-basal salts solution was aseptically added

[illegible] the cells were resuspended by scraping with a flamed wire loop, then mixed with a sterile 1.0 ml pipette. One milliliter from each beaker was used for ten-fold dilution series in sterile, LF-basal salts solution for the purpose of spread-plate counts. Of the remaining volume, 0.1 ml was aseptically pipetted and spread plated onto agar plates. Four plates were spread for each dilution.

Carbon Starvation

The same washed LF-TSS cell cultures were used for these experiments as for the desiccation experiments. Cells were starved in 50.0-ml, Kimax screw-cap test tubes. The tubes were autoclaved empty, and 8.0 ml of sterile, LF-basal salts solution were later added aseptically to each. The pH of the starvation solution was 7.2. A 2.0-ml volume of each cell type was then added to each of three tubes. The tubes were incubated horizontally on a 130 rpm rotary shaker at 30°C for 9 days. For enumeration of cells surviving the starvation treatment, 0.1 ml from each tube was aseptically spread plated onto each of four SNF-Congo Red plates. Ten-fold dilution series in sterile LF-TSS salts were also prepared and enumerated by spread plating.

Microaerobic Incubation

The same washed BMB-grown cells were used for these experiments as for the previous experiments. The incubation medium consisted of LP-basal salts solution containing Bacto-Agar (Difco). The agar was added to give a final concentration of 0.05% (wt/vol) per flask after cell addition. The basal salts solution and agar were dissolved by boiling, then 23.5 ml were added per 125-ml Erlenmeyer flask. The flasks were autoclaved, and concentrated sterile LP buffer and biotin were added aseptically soon after autoclaving and before cell addition. A volume of 6.0 ml of encapsulated cell suspension was added to each flask. Flasks were prepared in triplicate and incubated in stationary position at 30°C.

Aerobic Incubation

The BMB-grown, encapsulated cell inoculum for this and all following experiments was harvested, washed twice in sterile deionized water, and resuspended in a third volume of sterile deionized water to give a final OD_{560} of 0.23 to 0.25. Lawns of 58 to 66 days of age were used as inocula. For the incubation solution, basal salts were dissolved in concentrated amounts to give their final, correct concentrations after aseptic additions of LP buffer, biotin, carbon, or nitrogen sources and cells. The biotin was aseptically added as a 100X concentrated stock solution, and all other

additions to the LP-basal salts solution were added as 10× concentrated, sterile stock solutions that were sterilized by autoclaving.

The sugars employed were D-glucose (Difco), sucrose (Difco), and D-fructose (Calbiochem, San Diego, CA). All were prepared as separate 4.37% (wt/vol) stocks in deionized water. The organic acids were succinic acid (Fisher) and DL-malic acid (Sigma). Each acid was prepared as a separate 4.37% (wt/vol) salt stock that was neutralized to pH 7.0 with 10 M KOH before being brought to final volume. The nitrogen sources were reagent grade KNO_3 and $(NH_4)_2SO_4$. The KNO_3 was prepared as a separate 0.765% (wt/vol) stock, and the $(NH_4)_2SO_4$ was prepared as a separate 0.5% (wt/vol) stock.

The same type of screw cap tubes used in carbon starvation studies were used in these studies. Each empty, sterile tube had aseptically added to it 8.0 ml of sterile, LP-basal salts solution plus biotin and a single carbon or nitrogen source. Sometimes cells were incubated in LP-basal salts solution with biotin, but without carbon or nitrogen sources. Then 2.0 ml of water-washed cells were aseptically added to each tube. Treatments for each carbon or nitrogen source were done in triplicate. Tubes were incubated horizontally on a 130 rpm rotary shaker at 30°C. The pH of amended buffer incubations ranged from 7.1 to 7.2.

Soil Dialysis Incubation

These cultures were prepared [illegible] of the method of Gonzalez-Lopez and [illegible]. The soil employed was Arredondo fine sand (Grossarenic Paleudult, loamy, siliceous, hyperthemic), obtained from the top 20 cm of a soil profile. The soil was air dried, then the fraction that would pass through a 0.250 mm sieve was recovered for further use.

Spectrapor membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) of 40 mm diameter and 6,000-8,000 molecular weight cutoff was used. Tubing was cut into approximately 25 cm long pieces and soaked in a solution of 1.0% (wt/vol) $NaHCO_3$ in which was dissolved 0.005% (wt/vol) EDTA. The pH of this solution was reduced to 7.5 with 1 M HCl before making it up to final volume. The pieces of dialysis tubing were soaked and then boiled in this solution for at least 10 min to remove substances that might harm bacterial cells. The tubing was then rinsed five times in changes of deionized water and resuspended in a sixth wash. One end of each piece of tubing was then knotted once.

Five grams of sieved soil were added to each piece of tubing by inserting a glass funnel in the open end of each piece and pouring the soil in. The soil was washed down and evenly distributed in each piece of tubing by adding about 10 ml of deionized water to the open end with a squirt bottle. The open end of each tube was then knotted once,

allowing empty space [illegible] help reduce stress due to expansion of the tubing [illegible] autoclaving. Each piece of tubing was then rocked gently and agitated while holding one end in each hand, to make sure the soil had become thoroughly wetted. Each piece of tubing was then added to 45 ml of deionized water in a 250 ml Erlenmeyer flask and autoclaved for 25 min. The pH of the sterile, equilibrated soil solution surrounding the sterile, intact tubing was 6.1. For cell incubations, 0.5 ml of 100X sterile biotin was aseptically added, followed by 5 ml of water-washed cell suspension. Triplicate flasks were incubated at 130 rpm at 30°C for 24 to 48 hours. The friction between fine soil particles and dialysis tubing caused bags of soil to break during longer shaken incubations.

Tris-EDTA Treatment

A solution of 30 mM EDTA dissolved in 0.05 M Tris-HCl was prepared, and its pH adjusted to 8.4 with 10 M KOH. A solution of 0.05 M Tris-HCl was also prepared and similarly adjusted to the same pH. Both solutions were sterilized by autoclaving. For lysis experiments, 4 ml of either solution were aseptically added to 4 ml of water-washed, encapsulated cells in 50-ml screw cap tubes, to give a final concentration of 0.025 M Tris-HCl alone or in combination with 15 mM EDTA. Tubes were prepared in triplicate and incubated as for the other tube assays.

Chloramphenicol Treatment

A 0.2% (wt/vol) solution of chloramphenicol (Sigma) in deionized water was sterilized by passage through a 0.2 um pore size, Nalgene filter unit. Various volumes of this stock solution were added to water-washed, encapsulated cells in sterile Nutrient Broth (Difco), having a final concentration after all additions of 0.8% (wt/vol). Usually 2.0 ml of cells were added to these tubes. Incubation was in 50-ml screw cap tubes, under the same conditions for tubes as described above.

Phase-Contrast Microscopy and Photographs

These were the same as in Chapter II.

Results

Desiccation Resistance

Neither the vegetative nor the encapsulated cells displayed significant resistance to the drying method employed. Virtually all cells lost viability during the 8 days of desiccation. Table 3-1 gives the results of two separate desiccation experiments. No statistical analyses were performed, because even if some difference could be revealed between vegetative and encapsulated cells, the survival of either was so poor as to be negligible

Table 3 [illegible] of Azospirillum lipoferum Sp RG6xx.

Experiment Number	Cell type	Initial CFU ml^{-1} ($\times 10^5$) dried[a]	Beaker CFU ml^{-1} final 1	2	3	% survivors[b]
1	Vegetative	0.72 (0.03)	0.0 (0.0)[c]	22.5 (13.0)	5.0 (5.0)	0.03
	Encapsulated[d]	0.42 (0.06)	92.5 (37.7)	55.0 (28.7)	---[e]	0.35
2	Vegetative	0.14 (0.03)	10.0 (7.1)	0.0 (0.0)	0.0 (0.0)	0.05
	Encapsulated[f]	1.34 (0.10)	2.5 (4.3)	25.0 (43.0)	40.0 (17.3)	0.03

[a]Averages of colony forming units (CFU) of four spread plates. Values in parentheses are standard deviations.

[b]Average of all beakers available.

[c]Averages of four spread plates. Values in parentheses are standard deviations

[d]75-day-old cells.

[e]Beaker was lost

[f]53 day-old cells.

Starvation Resistance

After 5 days of starvation in carbon-and-nitrogen-[illegible] LP-basal salts solution lacking biotin, the encapsulated cells retained 24 to 27% viability. On the other hand, vegetative cells multiplied several-fold and retained viability (Table 3-2). It was interesting that, although two different initial densities of vegetative inocula were used, each seemed to stabilize at about 10^6 CFU/ml after 9 days of starvation.

Germination Experiments

Table 3-3 gives a summary of the germination experiments involving semisolid agar, buffered salts solution containing single carbon or nitrogen sources, and soil dialysis flasks. The soil dialysis flasks and combined-nitrogen incubations all resulted in germination of encapsulated cells. Within 18 hours, the majority of cells in these treatments were extremely motile, vegetative cells. By this time the motile cells in soil dialysis flasks had lost most or all visible deposits of phase-bright PHB granules. The PHB granules were usually still visible in the nitrate and ammonium treatments after 24 hours, but were markedly reduced in size from those in the initial inoculum. Within 72 hours the extremely motile cells in the combined-nitrogen treatments assumed the totally phase-dark appearance of motile cells in soil dialysate. Figure 3-1

Table 3-2. Starvation resistance of Azospirillum lipoferum Sp RG6xx

Experiment Number	Cell type	Initial CFU ml^{-1} (X 10^6)[a]	Final CFU ml^{-1} (X 10^6)[b]	% Initial CFU ml^{-1}
1	Vegetative	0.72 (0.03)	1.02 (0.26)	142.04
	Encapsulated[c]	0.42 (0.06)	0.11 (0.01)	26.61
2	Vegetative	0.14 (0.03)	1.02 (0.22)	751.23
	Encapsulated[d]	1.34 (0.10)	0.33 (0.18)	24.22

[a]Averages of colony forming units (CFU) of four spread plates. Values in parentheses are standard deviations

[b]Averages of three starvation tubes, with four spread plate counts per tube. Values in parentheses are standard deviations

[c]75-day-old cells.

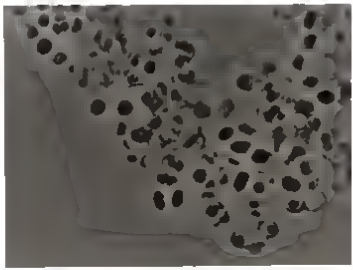
[d]53-day-old cells.

Table 1. Germination of encapsulated cells of *Azospirillum lipoferum* RG6xx to [illegible] incubations

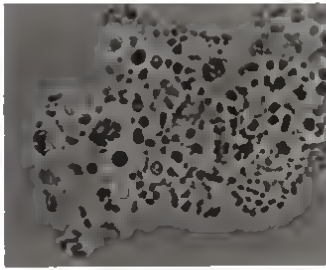
Treatment	Germination[a]
Soil dialysate	+
NO_3^-	+
NH_4^+	+
Glucose	-
Fructose	-
Sucrose	-
Malate	-
Succinate	-
Aerobic, low phosphate, basal salts solution	-
Microaerobic, low phosphate basal salts solution	-

[a] + denotes the majority of cells present becoming motile and depleting their visible poly-β-hydroxybutyrate reserves within 24 hours.

Figure 3-1 Encapsulated cells of Azospirillum lipoferum Sp RG6xx that have undergone germination in low phosphate-basal medium solution with combined nitrogen a) Cells from a 29-hour nitrate incubation. Note germinated vegetative cell at left of an empty capsule 1,000X magnification. Bar equals 4.0 µm b) Cells from a 29-hour ammonium incubation. Note germinated vegetative cells, empty capsules and poly-β-hydroxybutyrate-rich cells remaining in the floc. 1,000X magnification. Bar equals 4.0 µm.



a



b

cells from 29-hour incubations in nitrate (Figure [illegible]) and ammonium (Figure 3-1b), and Figure 3-2 shows cells from a 29-hour incubation in soil dialysate. As is apparent in these photographs, most encapsulated flocs had germinated, retaining their general shape after most of their cells had left them. The pH of inoculated soil dialysis flasks rose to 6.4 to 6.5 within 24 hours after inoculation.

Encapsulated flocs suspended in buffered-salts solution produced a few motile, ovoid to peanut-shaped cells that remained swollen with intracellular PHB deposits. This was also true in the semisolid agar flasks and in the buffered-salts solution tubes containing single carbon sources. Sometimes these PHB-rich cells were as rapidly motile as the cells that germinated with combined nitrogen and soil dialysate. Usually, however, they moved slowly and were prone to long periods of twiddling before they actively moved off on a run. Although these treatments often resulted in large numbers of free, nonencapsulated cells, most of these individual cells were not motile. The original inocula did not contain even weakly motile cells and contained few individual cells. Figure 3-3 shows cells from a 29-hour incubation in buffered-salts solution containing glucose, an incubation where the flocs remained largely occupied with cells.

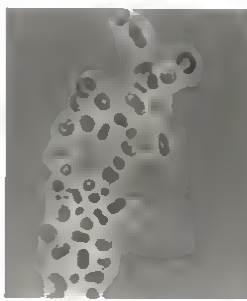
[illegible] ated cells of Azospirillum lipoferum Sp RG6xx that have undergone germination in soil dialysis flasks a) Cells from a 29-hour-old incubation. Note empty horseshoe-shaped capsules and phase-dark vegetative cells. 1,000X magnification Bar equals 4.0 µm. b) Other cells from a 29-hour incubation. Note empty horseshoe-shaped capsules, phase-dark vegetative cells and poly-β-hydroxybutyrate-rich cells remaining in the floc 1,000X magnification Bar equals 4.0 µm.

a

b

Encapsulated cells of Azospirillum lipoferum Sp RG6xx that have not undergone widespread germination in low phosphate-basal salts solution with glucose. a) Cells from a 25-hour incubation. A few empty capsules are present, but most capsules in the microfloc still retain their cells. 1,000X magnification. Bar equals 4.0 µm. b) Microfloc from a 25-hour incubation with several empty capsules. Note rounded cyst-like appearance of some cells remaining in the floc. 1,000X magnification. Bar equals 4 0 µm

a



b

After several weeks of incubation some morphological changes were observed for cells in the treatments that were scored as negative at 29 hours. Flocs that were incubated in aerobic, LP-basal-salts solution for 60-65 days had somewhat the same appearance as germinated flocs, in that most capsular spaces appeared empty or contained cells of unhealthy appearance. Less than 5% of the capsules in this extended incubation contained PHB-rich cells. The cells apparently had not germinated, since no individual vegetative cells were observed outside of the flocs. Cells apparently had depleted their visible PHB reserves and starved in place within their capsules. The addition of carbon sources seemed to reduce the extensive loss of visible PHB deposits. The number of cells within a floc that retained visible PHB deposits and an overall viable appearance after 47 days of incubation ranged from about 5 to 20% in aerobic, carbon-amended incubations. Often these cells appeared somewhat reduced in size, having contracted from the capsule boundary while retaining their rounded appearance. A few individual, motile, PHB-rich cells were also present in carbon-amended incubations at this time. The nitrate and ammonium incubations at 47 days consisted of empty capsules and nonmotile, phase-dark vegetative cells which had the appearance of stationary phase cells. After 60 to 65 days, the microaerobic incubations were similar in appearance to the aerobic, carbon-amended incubations, with

many PHB-rich cells [illegible] in capaules. [illegible] all the other extended in [illegible] only the microaerobic treatment contained numerous, motile, phase-dark vegetative cells.

Tris-EDTA Treatment

When encapaulated flocs were incubated in Tris-EDTA solution, the only observable effect was diasolution of macroscopic flocs within 24 hours. To the naked eye, tha cell suspenaions appeared more evenly turbid and nonfloc-culated than those from any other treatment. When viewed by microscopy, however, microflocs were still present. They were of about the same aize range as microflocs in other treatments, and had about as many cells remaining in their capaulea. Few motile oells were observed in this treat-ment. There were not noticeably fewer cells within capaules than in the other treatments where overall germination did not occur. The same results were obtained when encapsulated flocs were incubated in Tria alone.

Effect of Chloramphenicol

The addition of 50 µg chloramphenicol/ml to nutrient broth prevented growth for at least 36 houra, whereaa nutrient broth tubea without the antibiotic became turbid within 18 hours with the same inoculum level. Even in the presence of chloramphenicol, however, a few weakly motile,

PHB-rich cells were observed. Each was rota[illegible] its own long axis without moving off on runs

Discussion

The desiccation resistance assay used in these studies involved rapid drying of the cells. Mature, encysted forms of some prokaryotes are much better able to withstand drying on glass surfaces than their vegetative counterparts (Whittenbury et al., 1970a; Tudor and Conti, 1977). Filter membranes are often used as a surface upon which cells are slowly dried in desiccation resistance assays (Socolofsky and Wyss, 1962). In such experiments (data not shown), vegetative cells of Azospirilla sometimes survive slow drying on membrane filters without appreciable die off, as do encapsulated cells. Webb (1965) has pointed out the difficulties of using membrane filters in such assays. It was thought that glass surfaces would be easier to use, with less inherent hydrophilic behavior than membrane filters. Rapid drying of cells within a day's time or less usually causes a rapid and nearly complete die off of vegetative cells for many genera (Robinson et al., 1965, Whittenbury et al., 1970a; Tudor and Conti, 1977). The results of the work reported here show no apparent significant difference in response to rapid drying between vegetative or encapsulated cells of A. lipoferum Sp RG6xx. This is in agreement with studies where capsules have not enhanced the ability of

[illegible] cation (Dudman, 1977, Lowendorf, 1980).

Other studies have found enhanced desiccation resistance for some encapsulated forms of Azospirilla. Such forms have been called cysts (Lamm and Neyra, 1981; Papen and Werner, 1982; Sadasivan and Neyra, 1985). In the first study, however, cyst-like forms were slowly dried while vegetative cells were rapidly dried, so a true comparison of desiccation resistance between forms seems questionable. In the last study, flocs of cells were stored without desiccant for long time periods, and some cells within pieces of flocs were shown to remain viable for up to six months. But whether this reflects a true desiccation resistance of single cells, or only an ability to withstand drought in the presence of hydrophilic polymers (Jagnow, 1982), is unclear. Perhaps the best studies were done by Papen and Werner (1982), where vegetative cells were found to almost completely die out rapidly on dried membrane filters, whereas encapsulated, nonmotile cells survived this treatment in high numbers.

Azospirilla may undergo extensive morphological changes, including accumulation of PHB and capsular material, as only a first step towards becoming mature cysts. Morphologically cyst-like azospirilla have exhibited variable responses to desiccation (Lamm and Neyra, 1981, Papen and Werner, 1982). In the studies reported here, many

cells in micr[illegible] a cyst-like appearance. However, cells in microf[illegible] survived desiccation no better than nonencapsulated vegetative cells. Encysting bacteria may gradually mature, with their resistance to environmental stress increasing with time although their morphology appears cyst-like throughout. Modification of the cell membrane may be the true key to stress-resistance of these forms, as may also be true of Azotobacter spp. cysts (Reusch and Sadoff, 1983). Immature cysts of azospirilla may mature best if they are removed from the growth medium and/or slowly dried. Sadasivan and Neyra (1985) removed their encapsulated azospirilla from broth and dried the flocs slowly, and possibly obtained desiccation resistant forms. Similarly, Lamm and Neyra (1981) obtained desiccation-resistant azospirilla by allowing the agar of lawns to dry slowly.

There was a significant difference in response to carbon and nitrogen starvation between vegetative and encapsulated cells of A. lipoferum Sp HG6xx. After 9 days, only about 25% of the original encapsulated inoculum retained viability. The cell densities used in these starvation experiments were less than 10^6 CFU/ml, so microscope observations were not performed. However, the microscope observations of extended germination incubations with higher cell densities indicated that the majority of PHB rich cells within flocs will eventually deplete their PHB reserves and

apparently lose via[illegible] or exogenous carbon. Most encapsulated [illegible] flocs do not become motile when confronted with [illegible] or exogenous carbon. From 5 to 25% of cells [illegible] flocs retained their PHB after extended incubation and [illegible] underwent reduction in size within their capsules. One interpretation of this size reduction is that the cells were undergoing maturation into physiologically mature cysts. In contrast to the encapsulated cells, two different densities of vegetative inoculum increased several fold during the same 9 days of starvation to give about 10^6 CFU/ml. This apparent multiplication to a certain cell density and continued viability of vegetative cells faced with starvation have a precedent in studies of other bacteria, such as Rhizobium japonicum (Crist et al., 1984).

In a recent study, Tal and Okon (1985) reported that vegetative, PHB-poor cells of A. brasilense strain Cd died off to about 7% of their initial numbers after 130 hours of starvation in sterile, 0.06 M potassium phosphate buffer. In comparison, PHB-rich cells proliferated 2.3-fold over the same time span. Their experimental conditions differed from conditions reported here not only in using a different bacterial species but also in incubating the cells in phosphate buffer alone, without other salts. Both of their cell types were apparently nonencapsulated and actively motile, while the encapsulated cells in this study were not

motile. There are numerous starvation conditions that bacteria can be exposed to in vitro, and the response of different strains can vary widely under different conditions. It would be interesting to follow up on these initial studies of starvation resistance of azospirilla, to gain further insight into how they might survive in soil in the absence of plant material. It is possible that some azospirilla are able to enter into two types of dormancy (Marshall, 1980) in unfavorable soil conditions. If the cells have been experiencing balanced growth before they are starved of exogenous carbon, they might enter into exogenous dormancy. Such cells would be poor in PHB and might have no different morphology than growing vegetative cells, but their metabolism would be greatly reduced. If the cells have accumulated large amounts of PHB through dinitrogen fixation or extremely rapid uptake of carbon sources during growth on combined nitrogen, they might be prone to enter constitutive dormancy or an encysted state when faced with starvation.

Papen and Werner (1982) suggested that depletion of available oxygen was responsible in part for encystment of azospirilla in their studies. The low oxygen consumption of encysted cells may have allowed oxygen to diffuse back into the medium from the headspace, whereupon vegetative cells emerged from the capsular coats and resumed dinitrogen fixation. Because of their suggestion, in this study

[illegible] flocs were incubated microaerobically in semi-solid agar, as well as aerobically in shaken broth tubes, to give the cells different oxygen regimes. There was no observable difference in response between flocs incubated microaerobically or aerobically in buffered salts solutions with or without single carbon sources. In each of these treatments, a few weakly motile, PHB-rich cells were observed within 18 hours, and they persisted for up to 10 days. Half of the capsular spaces in some flocs were empty, but most flocs retained the majority of their cells within capsules. Unlike mature Azotobacter spp. cysts, encapsulated cells of this strain did not become synchronously motile when exposed to carbon sources (Loperfido and Sadoff, 1973). Phosphate-buffered-salts solutions and buffered-salts solutions containing sucrose, a nonmetabolizable carbon source for this species (Tarrand et al., 1978), produced about as many motile cells as did metabolizable carbon sources. Like mature Azotobacter spp. cysts, encapsulated cells of this strain seemed unable to mobilize their PHB reserves in unamended buffered salts solution to enable germination and widespread motility (Loperfido and Sadoff, 1973). Either wetting released cells from flocs and these cells became motile, or else the cells became motile and actively left flocs after wetting. Perhaps both events occurred. In any case, most individual cells were not motile in these incubations. The semisolid agar flasks

[illegible] though cells at the initial inoculum to be viable [illegible], but the cells remained dispersed throughout the [illegible] formed no pellicle. As a result, these treatments are listed as giving no germination (Table 3-3). A few cells seemed able to slowly mobilize their PHB reserves and become motile under these conditions, but the majority of cells remained in the floc, or once free from the floc, remained nonmotile. Motile cells continued to retain extensive visible deposits of PHB.

Undeniable germination occurred when the flocs of encapsulated cells were added to soil dialysis flasks, or to buffered-salts solution containing nitrate or ammonium. The uniformity of response among these treatments indicates that combined nitrogen in the soil dialysate was responsible for its germination effect. It also indicates that most of the cells in the flocs were not similar to mature Azotobacter spp. cysts, which do not germinate in the presence of ammonium (Loperfido and Sadoff, 1973). The availability of combined nitrogen apparently prompted most of the cells in encapsulated flocs to mobilize their PHB reserves and return to an actively motile, vegetative state.

An interesting feature of these positive germination treatments was the persistence of nonmotile, PHB-rich cells within flocs even after 10 days of incubation. Some of these cells no longer possessed a plump appearance, and their PHB granules were dispersed irregularly within the

cytoplasm. Sometimes capsules seemed to contain PHB polyphosphate granules without any cytoplasm. Empty capsules, however, often possessed the 'horseshoe' shape typical of empty exines of mature Azotobacter spp. cysts (Lin and Sadoff, 1969).

The Tris-EDTA incubations did not produce any obvious expulsion of cells from capsules. The concentration of EDTA was about five times that which produces prompt expulsion of central bodies from mature Azotobacter spp. cysts (Lin and Sadoff, 1969). As mentioned earlier, however, the macroscopic appearance of the inocula was rendered more evenly turbid by this treatment. Incubation in Tris buffer alone had the same effect as Tris-EDTA. The high pH of the treatments (pH 8.4) may have been related to the dispersive effect, along with the chelating effects of the Tris and the EDTA.

It seems that protein synthesis is necessary before encapsulated cells are able to become motile in large numbers. The chloramphenicol treatment did not prevent some free cells from spinning about their own long axes, however.

Based on these tests, there appears to be little physiological similarity between mature cysts of Azotobacter spp. and most cells in the encapsulated flocs of A. lipoferum Sp RG6xx. Most of the cells in encapsulated flocs represent immature cysts, lacking desiccation resistance, but being largely nonmotile and unable to readily mobilize

their PHB reserves unless exogenous, combine[illegible] becomes available. If these cells are found to ha[illegible] low endogenous respiratory rate, it might further indicat[illegible] their state as nascent cysts.

Living cells may form structures that prove immediately useful for some functions. By chance these structures may also prove beneficial to the cells in other ways (Cairns-Smith, 1982). It is suggested that the microfloc of azospirilla are such structures. Their possible benefits were suggested in Chapter II. Four observations in this chapter deserve further comment. One is the great size difference between motile, vegetative cells of A. lipoferum Sp RG6xx and nonmotile, encapsulated cells. The encapsulated cells occupy much more volume. Secondly, Costerton et al (1981) suggested that most bacterial cells in nature assume two forms. Sessile forms surrounded by a capsule maintain a population on a surface and give rise to motile swarmer cells which colonize new surfaces. This is a good description of the conversion of encapsulated to motile forms of A. lipoferum Sp RG6xx. Thirdly, the ability of only 5 to 20% of cells within encapsulated floca to retain their viable PHB deposits over 65 days of aerobic nitrogen starvation may indicate the physiological diversity of cells within an encapsulated PHB-rich microfloc. Most cells may be poised to become motile or resume vegetative growth and may represent the cells that depleted their PHB reserves

[illegible] starvation and which finally lose [illegible] ility [illegible]ic remaining cells within the microfloc may include cells that eventually mature into cysts. Finally, the similar "horseshoe" appearance of some empty capsules of azospirilla and of germinated cysts of Azotobacter spp. may have some importance. More than one type of capsule may exist within an encapsulated microfloc of azospirilla, and some capsules may have proceeded further toward a cyst-coat composition than others.

A few encapsulated cells in flocs survived the desiccation treatment. A few cells within flocs also had the appearance of rounded, possibly mature cysts. Sometimes they broke free of flocs (Figure 3-4). They were never observed to be motile. If these are truly mature cysts, the problem remaining is how to convert most of the cells in a vegetative inoculum quantitatively into this form.

Figure 3-4. Rounded, possibly cyst-like cells of Azospirillum lipoferum Sp RG6xx, from a 29-hour incubation in low phosphate-basal salts solution with glucose. 1,000X magnification. Bar equals 4.0 µm.

CHAPTER IV
GENERAL CONCLUSIONS

The strains of azospirilla used in this study did not undergo quantitative morphological encystment when grown on nitrogen-free BHB agar. The strains of Azospirillum lipoferum synthesized exopolysaccharides more extensively than did the strains of Azospirillum brasilense. The A. lipoferum strains experienced unbalanced growth under these cultural conditions. They accumulated PHB and experienced unbalanced cell wall synthesis, as evidenced by the common formation of filaments and chains. Eventually the filaments or chains lost motility and accumulated capsular material. The final outcome was the formation of microflocs of encapsulated, PHB rich cells that often arose from only a few elongated cells. Some of the cells within these flocs had a cyst-like morphology. Environments with available nutrients having a high C/N ratio, such as the rhizosphere, may promote the formation of PHB and capsules by azospirilla. Some cells having these features may eventually form cysts. Cells in encapsulated microflocs may have some survival advantages that individual cells of azospirilla lack.

Encapsulated cells of Azospirillum lipoferum Sp RG6xx grown on nitrogen-free NHB agar were found to have far more PHB than cells grown in broth with combined nitrogen. Neither cell type displayed significant desiccation resistance. When faced with aerobic starvation for exogenous carbon and nitrogen, encapsulated cells of this strain died off after 9 days to about 25% of their original numbers. These survivors may have represented cells that were maturing into cysts. Vegetative cells grown with combined nitrogen multiplied several fold over the same period of starvation. This indicates that cells of this strain may not need to form cysts in nature to survive prolonged periods of starvation. Vegetative cells having reduced metabolic activity may survive such periods. Combined nitrogen promoted germination of nonmotile, encapsulated cells of this strain.

Although these studies failed to obtain cysts of azospirilla in high quantity, they may have provided some information of practical importance. The starvation studies suggested that encapsulated, PHB-rich cells of azospirilla are less active physiologically than motile, vegetative cells. Further studies might measure nitrogenase activity and plant growth substance production by azospirillia in relation to PHB deposition and capsule formation. Such studies should lead to an understanding of what physiological form of azospirilla is most beneficial to plant growth.

Inoculum production may then be designed to produce a
sustain the most beneficial physiological [illegible] in the root
zone

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I [illegible] that I have read this study and that i[illegible] opinio[illegible] conforms to acceptable standards of scholar[illegible] presentation and is fully adequate, in scope and quali[illegible] as a dissertation for the degree of Doctor of Philosophy

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stephan L. Albrecht
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

Stephen G. Zam
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

David J. Mitchell
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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